Alterations in Spinal Motoneurone Population and Muscle Architecture after Peripheral Nerve Repair : A Quantitative and Qualitative Study.

by

Joyce Gilmour B.Sc.

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Declaration

I hereby declare that all the work presented in this thesis is original and has never previously been presented for any other degree or professional qualification. The thesis has been composed entirely independently.

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Abbreviations

α	Alpha
Ache	Acetylcholinesterase
ATPase	Myosin adenosine triphosphatase
Control	Contralateral control
EDL	Extensor digitorum longus
γ	Gamma
H & E	Haematoxylin and Eosin
HRP	Horseradish peroxidase
LHS	Left hand side
MG	Muscle graft
NADH-TR	Nicotinamide adenine dinucleotide tetrazolium reductase
NC	Nerve crush injury
N-CAM	Nerve cell adhesion molecule
NG	Nerve graft
NN	Direct epineurial suture
N-N suture	Direct epineurial suture
NS	No significant difference
RHS	Right hand side
RSE	Relative standard error
SD	Standard deviation
SDH	Succinate dehydrogenase
Sol	Soleus
TA	Tibialis anterior
TMB	Tetramethyl benzidine
Vv	Volume fraction

Abstract

The aim of this study was to assess the changes which occurred in the spinal motoneurone pool and in the target muscle after the repair of a specific peripheral nerve, using several clinically appropriate surgical techniques. The motoneurone pool relating to a single muscle was assessed at 50, 100, 200 and 300 days after repair, by means of retrograde axonal transport of the neural tracer horseradish peroxidase. The results indicate that whilst a small portion of the motoneurone population dies after peripheral nerve surgery, the majority of the anterior horn cells appear to have the ability both to survive nerve transection and to form new functional connections after repair. The number of motoneurones associated with the extensor digitorum longus (EDL) after the transection of the sciatic nerve was significantly less than normal, however there was no significant difference in the number of motoneurones labelled after each of the methods of repair. After axonotmesis there was no significant difference in the number of labelled motoneurones compared to normal. There was no significant difference between the mean minimum cell diameters of labelled motoneurones in the ventral horn of the spinal cord after each of the methods of repair.

There were alterations in the size, shape, morphology and cytochemical architecture of the fibres of the target muscles after the injury and repair of the sciatic nerve. Although these changes were more marked after the transection of the nerve compared to the less severe crush injury, there was little or no difference in the architecture between the groups which had been repaired by the different methods. One of the most striking features after the transection and repair of the

sciatic nerve was the appearance of groups of fibres of the same type, as opposed to the random distribution seen in normal muscle or indeed in muscle which had been reinnervated after a nerve crush injury.

These results suggest that the extent of cell loss and the changes in muscle fibre architecture are influenced by the type of injury rather by the method of repair. The main problems of functional recovery found after peripheral nerve repair such as muscle weakness, uncoordinated movement and the false localization of stimuli are not a consequence of changes in the number of spinal motoneurones, the degree of reinnervation attained or of changes in the muscle fibre architecture. It is likely that the limited motor and sensory function recovered after the transection and repair of a peripheral nerve is a result of the inaccuracies which arise in the process of reinnervation.

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Introduction.

The history of attempts to repair gaps in peripheral nerves goes back almost to the dawn of Listerian surgery (Seddon 1975). Indeed, in 1886, Lister himself suggested that connecting the ends of a cut nerve by means of several very fine catgut stitches would provide a channel along which new nerve tissue may develop (Cameron 1944). Great advances in both knowledge and technology have been made since then, most notably around the time of the two World Wars. Despite these advances, poor recovery of function after peripheral nerve injury and repair is still a problem which is ill understood by surgeons and scientists alike (Glasby 1990).

The extent of anatomical and physiological changes which occur in the motor unit after peripheral nerve damage depends on the severity of the injury. Nerve injuries are usually defined either by the classification devised by Seddon (1943, 1954, 1972) or by Sunderland (1951, 1978), although a variety of other classifications has been devised based on the structural and functional alterations of the nerve fibres (Lundborg 1988). Seddon defined three different types of nerve injury, namely neurapraxia, axonotmesis and neurotmesis. Neurapraxia results in the interruption of nerve conduction at the site of injury but axonal continuity is maintained between the cell body and the target organ. Neurapraxia results in motor paralysis but some sensory and sympathetic functions are maintained (Seddon 1972). Neurapraxia corresponds to the first degree injury of Sunderland (1978). Axonotmesis is a crush injury to the nerve which is sufficiently severe to

cause distal degeneration but the sheath and supporting structures of the nerve remain intact. The injury is severe enough to result in Wallerian degeneration of the distal portion of the nerve (Lundborg 1988). Axonotmesis corresponds to the second degree injury of Sunderland (1978). Neurotmesis is the total severance of the nerve whereby there is a loss of continuity of axons, endoneurial tubes, perineurium and epineurium. Like axonotmesis the injury results in Wallerian degeneration of the distal portion of the nerve. Neurotmesis corresponds to the fifth degree injury of Sunderland (1978). Sunderland defined two further types of injury, namely third and fourth degree injuries, which are more severe than axonotmesis but less so than neurotmesis. A third degree injury results in the loss of continuity of axons and endoneurial tubes while the perineurium is still intact whilst a fourth degree injury results in the loss of continuity of the perineurium but the epineurium is preserved. A fifth degree injury results in the loss of continuity of the entire nerve trunk (Sunderland 1978).

1.1 The response of the motor unit to damage.

When a peripheral nerve is injured by means of either axonotmesis or neurotmesis, the distal portion of the nerve undergoes Wallerian degeneration. Consequently, a characteristic sequence of events is initiated in the cell body, the nerve and in the target muscle. These events promote effective regeneration (Slater and Harris 1988).

a. The response of the cell body to neuronal damage.

When a peripheral nerve is severed, the nerve cell bodies in the motoneurone pool undergo a series of reactive changes known as chromatolysis. These changes

begin within hours of the injury and are most pronounced between one and three weeks after axotomy (Lieberman 1974, Sunderland 1978, Peyronnard et al 1986). The process of chromatolysis involves both cytoplasmic and nuclear morphological changes, which have been described in detail by Lieberman (1974). Chromatolysis usually begins with the swelling of the cell body (Barr and Hamilton 1948, Cragg 1970, Lundborg 1988), followed by the displacement of the nucleus from its central position to the periphery (Sunderland 1978, Lundborg 1988). The larger Nissl bodies tend to lose their colour and subsequently disintegrate to give a fine powdery appearance and clear areas of cytoplasm lacking Nissl bodies (Cragg 1970, Sunderland 1978). Indeed it is the loss of colour in the Nissl bodies that gave the process of chromatolysis its name, chroma meaning colour and lysis meaning loosing (Lieberman 1974).

The early signs of chromatolysis are followed by an anabolic reaction which involves an increase in RNA and protein production (Cragg 1970, Lundborg 1988). There is not only an increase in the level of RNA in the nucleolus of the damaged neurone but also an increase in the rate at which it passes out into the cytoplasm. Similarly, the rate of protein synthesis increases together with the rate at which it disappears from the cell body (Lundborg 1988). It is assumed that the movement of the proteins away from the cell body is the result of their migrating down the axon to aid in the regeneration process (Cragg 1970). There is an increased number of satellite (glial) cells around the cell body of a damaged neurone (Cragg 1970, Sunderland 1978).

The speed of onset and the exact nature of the chromatolytic changes which

take place in an injured neuronal cell body depend on a variety of factors such as the age and species of the subject, the nature of the injury, the proximity of the lesion to the spinal cord and the cell size (Cragg 1970, Sunderland 1978, Carlson, Lais and Dyck 1979, Schmalbruch 1987b, Lundborg 1988). The majority of cell bodies associated with damaged neurones will show signs of chromatolysis, however not all of these affected cells will subsequently die. It is not known why the stimulus of axotomy causes chromatolysis in some cell bodies and not in others of the same functional group (Sunderland 1978, Slater and Harris 1988). Similarly it is not known why some cell bodies subsequently die while others make a complete recovery (Slater and Harris 1988). There has been a variety of proposals put forward to explain what triggers the process of chromatolysis in damaged neuronal cell bodies. The theories suggested thus far include the depolarization of the cell by the cut end of the axon (Cragg 1970). When the nerve is transected the continuity of the cell membrane is lost allowing a sudden influx of sodium ions into the cytoplasm. This influx of sodium ions results in the depolarization of the cell. Alternative theories suggested as a trigger for chromatolysis include the loss of action potentials, the breakdown of the blood-nerve barrier, the increased loss of axoplasm and mitochondria, the loss of trophic substances coming from the periphery and the loss of a repressor substance (Cragg 1970, Sunderland 1978, Lundborg 1988). Several of these effects are present together after most forms of nerve injury and this is thought to result in the variety of responses found in different situations (Cragg 1970).

Most of the changes associated with chromatolysis are reversed if the

regenerating axons make functional contact with a muscle (Slater and Harris 1988). The earliest signs of recovery are the return of the nucleus to the centre and the reappearance of compact aggregates of endoplasmic reticulum (Sunderland 1978). Subsequently the cell returns to its normal size and shape as excess water leaves it (Sunderland 1978). Cells which do not recover from chromatolysis and are therefore destined to degenerate, can persist in a shrunken, chromatolysed state for several months (Sunderland 1978). These cells will subsequently undergo disintegration and phagocytosis (Torvik 1972). Consequently, after the injury and repair of a peripheral nerve there is a variety of changes seen in the motoneurone pool in the spinal cord.

b. The response of the nerve to damage - degeneration.

When a peripheral nerve is cut, the severed distal ends of the fibres degenerate (Mark 1969) since they are separated from the cell body which maintains them. The first part of the axon to break down is the synaptic terminal (Slater and Harris 1988). Subsequently the distal portion of the axon degenerates, along with its myelin sheath, in a process known as Wallerian degeneration (Waller 1850, Seddon 1975, Lundborg 1988). A similar degeneration process takes place over a short distance in the proximal stump, the extent of the retrograde degeneration being dependent on the severity of the lesion (Seddon 1975, Sunderland 1978, Lundborg 1988). The degenerating material is removed by macrophages, from the endoneurium, and Schwann cells which become temporarily phagocytic (Weiss 1944b, Slater and Harris 1988). The diameter of the tubes in the distal segment of the nerve become greatly reduced because of

shrinkage and collagen deposition (Sunderland and Bradley 1949, 1959).

c. The response of the nerve to damage - regeneration.

Whilst the distal segment of the nerve is degenerating the proximal segment initiates the regeneration process. The surviving Schwann cells begin to proliferate and become motile (Lundborg 1988). Axon sprouts begin to emerge from the proximal stump and grow between the cords of Schwann cells which have formed along the endoneurial tubes (Landon 1976, Ham 1987, Lundborg 1988). If the basal lamina tubes remain intact, as when the nerve has been crushed, they provide a direct channel for axonal growth from the proximal to the distal stump (Seddon 1975, Sunderland 1978, Lundborg 1988, Slater and Harris 1988), thus the regenerating axons are guided back to the periphery. If the nerve is cut and the ends retract, the growing sprouts rely on the Schwann cell cords to help guide them across the gap (Landon 1976, Ham 1987, Lundborg 1988). In such situations, there is very little chance that the growing sprouts will find their way back to the corresponding channels in the distal stump (Mark 1969, Sunderland 1978). This may lead to the regenerating axons terminating blindly in surrounding scar tissue or entering a foreign tube which will then conduct it to a functionally unrelated end organ (Sunderland 1978). The method of repair implemented has an effect on the degree of specificity obtained by the regenerating axons (Brushart et al 1983) but this topic will be discussed later (see "Factors influencing the quality of motor recovery"). The overall effect of this disorganisation in directional growth at the junction of the proximal and distal stumps is to reduce the fibre population available for reinnervation and to distort the pattern of innervation. Both of these

factors have a serious and adverse effect on the restoration of function (Sunderland 1978). Despite these problems a reasonable degree of recovery of function can be attained, however it tends to be weak with poorer coordination compared to normal (Brushart and Mesulam 1980).

The nerve fibres which successfully reach the endoneurial tubes of the distal stump regenerate along the tubes, but at a slower rate than occurs when continuity of the tubes has been maintained (Sunderland 1978). There tends to be an increase in the number of fibres present in the regenerated nerve (Aird and Naffziger 1939, Gutmann and Sanders 1943, Mackinnon and Trued 1986, Myles and Glasby, 1991). This is due to sprouting which takes place but it does not reflect the degree of functional recovery (Myles and Glasby, 1991). The regenerated nerve fibres tend to be smaller in diameter and have thinner myelin sheaths than control nerves (Myles and Glasby, 1991).The extent of the reduction in fibre diameter is dependent on the method of repair (Myles and Glasby, 1991).

Schwann cells have a crucial role in the process of axonal regeneration. They are responsible for protecting, supporting and remyelinating the regenerating axon sprouts in addition to providing guidance back to the periphery. It is thought that the poor results obtained following peripheral nerve repair using either long grafts or non neural tissue grafts is caused by the lack of Schwann cells (see Methods of Peripheral nerve repair).

d. The response of muscle to neuronal damage - denervation.

When the nerve supplying a muscle is cut, nerve-stimulated contractability of the muscle is lost and atrophy ensues (Jaweed et al 1975). Normal muscle fibres

are polygonal in shape as a consequence of their close packing within the fascicles, however as the individual muscle fibres atrophy the polygonal shape is lost and a more round or oval shape is assumed. As atrophy proceeds there is a corresponding increase in the amount of fat and connective tissue found between the muscle fibres (Gutmann and Young 1944, Sunderland and Ray 1950, Grieve et al 1991). The denervated muscle fibres undergo structural changes such as the development of an angular shape, granular or 'moth-eaten appearance', centrally located nuclei and longitudinal splitting. These features of denervation will be considered fully in chapter 6.

e. The response of muscle to neuronal damage - reinnervation.

When regenerating axons reach the denervated muscle reinnervation takes place. Neuromuscular transmission is restored and there is a reversal of the effects of denervation. The structural and functional changes seen in reinnervated muscle are considered fully in chapter 6.

1.2 Methods of peripheral nerve repair.

Successful reinnervation of the muscle can occur without surgical intervention after injuries which involve axonotmesis. Since, after axonotmesis, the basal lamina tubes remain intact, they guide the regenerating axons back along their original path resulting in very accurate reinnervation (Seddon 1975, Lundborg 1988). This, together with the fact that there is minimal muscle fibre atrophy following a crush injury, because of the minimal delays in the regeneration process, results in very good functional recovery (Bowden and Gutmann 1944).

Neurotmesis involves the severance of a nerve, the lesion is complete in

every sense. If no formal repair is implemented, then the regenerating axon sprouts will grow aimlessly from the proximal stump resulting in the formation of a large tangled mass of axons, fibroblasts and Schwann cells - a neuroma (Sunderland 1978). A similar, but smaller mass may also form on the distal stump and is termed a glioma (Young 1942, Weiss 1944). The formation of a neuroma will usually prevent the re-establishment of functional connections with target organs, in addition to causing a variety of other problems including severe pain for the patient. There is a chance that the neuroma and glioma will meet in the gap between the cut ends thus creating a potential pathway for nerve regeneration (Abercrombie and Johnson 1942, Kline, Hayes and Morse 1964), however the chances of this happening are remote (Glasby et al 1986). Indeed Sunderland (1978) states that recovery after an untreated fifth degree injury is negligible because of severe retrograde effects, the failure of many neurones to survive and the failure of the majority of regenerating axons to reach the endoneurial tubes of the distal stumps. Hence, if a peripheral nerve injury involves the severance of the nerve then recovery of function is greatly enhanced by surgical repair.

If the proximal and distal stumps are in very close proximity and the severed ends are not damaged, then the simplest form of repair is direct epineurial suture. The direct epineurial suture involves suturing the epineurium of the proximal and distal stumps together, whilst avoiding stretching or twisting of the nerve. Landmarks such as longitudinal epineurial blood vessels and fascicular pattern of the cut ends are used to ensure correct orientation of the stumps prior to suture (Lundborg 1988). An alternative form of repair is the individual fascicular suture whereby each fascicle is sutured to its corresponding distal fascicle. Individual

fascicular suture has been shown by Brushart et al (1983) to produce more accurate reinnervation than direct epineurial suture - see "Factors influencing the quality of motor recovery". However the technique has the potential disadvantage of surgical trauma, caused by the resection of the epineurium, added to the original injury (Lundborg 1988). If the gap in the nerve is so large that it would require tension to bring the two ends of the nerve together or if damaged ends have to be receded, then it is necessary to insert a graft into the gap (Millesi et al 1972a, Terzis 1975). Tension at the suture line is disadvantageous for axonal growth as it interferes with intraneural microvascular flow, increases scar tissue formation and decreases the quality of axonal regeneration (Millesi et al 1972a, Samii and Wallenberg 1972, Orgel and Terzis 1977, Miyamoto and Tsuge 1981a and b, Millesi and Meissl 1981, Lundborg 1988). The graft used to bridge the gap in the nerve can take the form of a nerve graft, a cable graft, a muscle graft or one of a variety of man made grafts, each of which has its own relative merits and pitfalls. The nerve, cable and muscle grafts all have the advantage of being autologous and so avoid the potential rejection problems associated with foreign grafting material. The use of nervous system tissue to form the graft is currently the most popular and logical method of repair. However there are two major disadvantages with this method, first a suitable non-essential donor nerve must be sacrificed to form the graft and secondly to obtain the donor nerve requires another operation on another part of the body which is time consuming for the surgeon and results in two (or more) scars for the patient. There is obviously a very limited supply of non-essential nerves which can be utilized, especially the larger diameter nerves (Glasby

1990). The implanted graft should ideally be of the same calibre as the recipient nerve, in order to optimize reinnervation (Seddon 1975). The use of cable grafts was seen as a way around this problem as this uses several strands of smaller diameter nerves grouped together in parallel and sutured into position to bridge the gap in larger nerves. The sural nerve is frequently used as the donor nerve as it is a small cutaneous nerve which can be sacrificed without creating a significant deficit (Glasby 1990). Although the resultant graft often works as well as a nerve graft, the cable graft has the disadvantage of being more awkward and time consuming to construct (Myles and Glasby 1991). A possible additional problem associated with both nerve grafts and cable grafts is that the large regenerating motor fibres may be constricted by the smaller diameter Schwann tubes present in the donor cutaneous nerve that form the graft (Weiss and Taylor 44b, Weiss et al 1945). However, Simpson and Young (1945) found that the larger regenerating nerves had no problem in inflating the tubes to a size which was sufficient for their effective function. The muscle graft avoids all of these problems as it is readily available, easy to construct, easy to insert, can be tailored to fit any size required and provides tubes which are larger in diameter than even the largest nerve fibre (Glasby 1990). It has an additional advantage in that it produces similar results to the cable graft despite the fact that it is simply sutured into the gap in the nerve whilst with the cable graft care is taken to align corresponding fascicles (Glasby, Gilmour et al 1990, Myles and Glasby 1991). The muscle graft has been shown to produce similar results to the more conventional methods of repair when the indices of morphology and functional recovery of the nerve are considered (Glasby

et al 1988a, Gattuso et al 1988b, Findlater et al 1990, Glasby 1992). However, it produces very poor results compared to the other methods, when considering the repair of long peripheral nerve defects (Hems and Glasby 1993). The failure of the muscle graft was thought to be caused by the basement membrane scaffold of the muscle being removed by inflammatory cells and being replaced by fibrous tissue before the regenerating fibres had a chance to grow through it. However it is likely that the length of the graft is also limited by the migratory powers of the Schwann cells (Nadim et al 1990, Hems and Glasby 1993).

1.3 Factors influencing the quality of motor recovery.

After regeneration, the peripheral motor and sensory systems are left impaired to a varying degree. Nerves which have sustained a 1st or 2nd degree injury tend to make a complete recovery with the full restoration of motor, sensory and sympathetic functions (Sunderland 1978). After a 3rd - 5th degree injury and nerve repair the patient is left with a residual disability of varying degree and distribution (Sunderland 1978). These disabilities may include loss of power, lack of sensation, impaired coordination and an inability of individual muscles to contract independently of one another.

Some of these disabilities may be attributed to the fact that the severance and suture of a peripheral nerve leads to muscle reinnervation that is abnormal both in degree and specificity (Brushart et al 1983). The fact that many anterior horn cells fail to regain peripheral connections, irrespective of the repair technique involved, indicates inherent limitations in the response to peripheral nerve injuries. The surgical technique does however influence the specificity with which muscles

are reinnervated by their original motoneurone pool (Brushart et al 1983). Individual fascicular suture leads to more accurate reinnervation than repair by means of a direct epineurial suture (Brushart et al 1983). In the case of a direct epineurial suture it is likely that the position of the cut fascicles will shift within the sutured epineurial sheath, thus leading to subsequent misalignment (Edshage 1964, Brushart et al 1983). The fact that individual fascicles are sutured together in the other type of repair, increases the likelihood of the regenerating axons entering the appropriate basal lamina tubes. Although this form of repair minimizes the chances of interfascicular disorganization, it does not prevent axon disorganization within the fascicle itself (Brushart et al 1981). The mismatch of regenerating axons and their appropriate basal lamina tubes occurs at the suture line between the proximal and distal stumps. When a repair involves the insertion of a graft, this further increases the chances of mismatch since the regenerating axons have to negotiate two sets of suture lines in the repair. The cable graft has an advantage over the muscle graft and nerve graft, in that it allows corresponding fascicles from the proximal and distal stumps to be aligned and joined by a strand of the cable. This ought to result in more accurate reinnervation than after the other forms of grafting in a similar way to the results found after individual fascicular suture and direct epineurial suture. However it has been shown that there is no difference between the different methods of grafting (Glasby, Gilmour et al 1990, Myles and Glasby 1991)

If the reinnervation is abnormal in specificity and the regenerating motor axons enter basal lamina tubes that lead to sensory branches, and *vice versa*, then

the problems are two-fold. Not only will they fail to make functional connections but they will also exclude the appropriate axons from the pathway (Brushart 1988). It has been suggested that although such mismatch is common after peripheral nerve repair, it is minimized by the preferential reinnervation of motor branches by motor axons. This preferential reinnervation is thought to be caused by a specific interaction between the motor axons and the Schwann cell tubes leading to the motor branch (Brushart 1988).

The functional consequences of inappropriate motor reinnervation will depend on the quality of sensory recovery and the degree of compensatory central reorganization (Brushart et al 1983). Central reorganization of patterns of activity compensate to a certain extent for the disturbances produced by the degeneration of neurones, loss of some axons and the cross shunting of others at the suture line. The extent of such central reorganization is largely unknown although the work of Sperry (1945) suggests that it is limited (Sunderland 1978). A great deal more is known about compensatory peripheral reorganization. After the severance of a peripheral nerve many neurones degenerate and die thus causing a deficit in the number of axons available to reform functional connections. This loss may be offset to some extent by the collateral sprouting of the axons of surviving cells. Such sprouting of a single regenerating axon can give rise to as many as 50 sprouts (Ranson 1912, Weddell 1942). Sprouting is much more extensive after the severance of a peripheral nerve than after a crush injury (Sunderland 1978). The most probable reason for this is that after a crush injury the full complement of axons usually regenerate to reform functional connections and hence extensive

sprouting is unnecessary. Although collateral sprouting increases the potential for recovery, by compensating for the loss of axons, it also runs the risk of reducing the quality of the recovery by overloading the neurones, adding to cross shunting of the axons at the suture line and multiple innervations of distal Schwann cell tubes (Sunderland 1978). The loss of some muscle fibres and the reduced efficiency of others may be offset by the hypertrophy of those fibres which have fully recovered. Incomplete reinnervation leaves some fibres uninervated but this tends to be overcome by collateral axon sprouting where the axons which have successfully reinnervated a muscle fibre will branch to innervate surrounding denervated fibres (Sunderland 1978). It is also possible that in the event of unsuccessful reinnervation of a muscle, another muscle may take on an additional role and compensate for the loss (Sunderland 1978).

The ability to overcome repair malalignement decreases with advancing age (Brushart 1991).

The quality of recovery after peripheral nerve surgery is notoriously unpredictable (Sunderland 1978). The failure to regain complete recovery of function may be due to one or many possible contributory factors such as characteristics of the nerve injury, faulty technique when uniting the severed ends, a delay in the repair, anatomical features in the nerve or to other factors which are beyond the control of the surgeon (Sunderland 1978). A great deal of research has been carried out into the changes which occur in the injured nerve itself, such as changes in the number of nerve fibres (Gutmann and Sanders 1943, Brushart et al 1983, Mackinnon et al 1985, Peyronnard et al 1986a, Glasby et al 1986c, Glasby

et al 1988a), fibre diameters (Gutmann and Sanders 1943, Aitken et al 1947, Cragg and Thomas 1964, Cragg 1970, Lieberman 1971, Hoffer et al 1979, Mackinnon et al 1985, Peyronnard et al 1986a, Glasby et al 1986c, Glasby et al 1988a, Gattuso et al 1988), myelin sheath thickness (Sanders and Whitterbridge 1946, Rushton 1951, Gattuso et al 1988), conduction velocities (Sanders and Whitterbridge 1946, Glasby et al 1988a, Gattuso et al 1988), development of mini fascicles (Gschmeissner et al 1990) and the specificity of regenerating fibres (Gutmann and Sanders 1943, Mark 1969, Brushart et al 1981, Brushart et al 1983, Brushart 1988, Brushart 1991). Each of these characteristic changes is influenced, to a certain extent, by external factors such as the type of injury (Hoffer et al 1979), the method of repair (Hoffer et al 1979, Brushart et al 1983, Brushart 1991), the timing of the repair (Glasby 1990), the distance of the injury from the spinal cord (Geist 1933, Barr and Hamilton 1948, Kline et al 1964a, Mackinnon et al 1985) and even by age and species (Geist 1933, Barr and Hamilton 1948, Kline et al 1964a, Kline et al 1964b, Lieberman 1974, Brushart 1991). It is not only the injured nerves which undergo such anatomical and physiological alterations, many changes also occur within the rest of the motor unit. It is the changes which occur in the cell bodies within the motoneurone pool of the spinal cord and within the target muscles which are the basis of the current study.

There has been limited consideration given to the possibility of changes in spinal motoneurones following peripheral nerve repair. In recent years some authors have considered changes which may occur in the motoneurone pool, such

as an alteration in the number (Lieberman 1971, Carlson et al 1979, Brushart and Mesulam 1980, McHanwell and Biscoe 1981, Brushart et al 1983, Schmalbruch 1984, Peyronnard et al 1986a, Peyronnard et al 1986b, Brushart 1990, O'Hanlon and Lowrie 1991), diameter (Ducker 1972, Burke et al 1977, Carlson et al 1979, Brushart et al 1983, Peyronnard et al 1986a, Peyronnard et al 1986b) or position of cells within the spinal cord (Brushart and Mesulam 1980, Brushart et al 1981, Nicolopoulos and Iles 1983, Brushart 1990) and changes in relative numbers of different cell types present (Kuno et al 1974, Brushart and Mesulam 1980, Nicolopoulos and Iles 1983, Peyronnard et al 1986a). These studies have looked briefly at factors which may influence the extent of these changes such as the type of injury, the age at which it is sustained and the subsequent treatment administered. To date no long-term comparative studies have been undertaken in order to assess the reactions of the motoneurone pool to different types of injury and repair.

A number of studies have recently been undertaken in order to assess differences which occur between normal muscles and denervated/reinnervated muscles. These studies have assessed changes in muscle fibre morphology (Johnson et al 1973, Swash and Schwartz 1984, Dubowitz 1985, Froes et al 1987), changes in enzymatic activity (Guth and Samaha 1969, Close 1972, Nemeth and Pette 1981, Pette et al 1983), different reactions of fast and slow muscle fibres (Close 1972, Jaweed et al 1975, Niederle and Mayr 1978, Lowrie and Vrbova 1984), fibre type ratios (Karpati and Engel 1968, Johnson et al 1973), recovery of motor function (Gutmann and Young 1944), effect of age of on recovery of the

muscle (Grieve et al 1991), effect of tendon transection and cross reinnervation (Close 1972, McLachlan 1983) and mapping of the motor units after reinnervation (Kugelberg et al 1970). Again no long term comparative studies have been undertaken in order to assess the reactions of the target muscles to different types of injury and repair.

There is a limit to the degree of influence that a surgeon can have over the outcome of peripheral nerve surgery since many of the factors which influence the success of the repair are out with his control. The method of repair implemented is an exception. The effect that the different methods of injury and repair has on the nerve itself has already been investigated thoroughly but it is very short sighted to restrict such research to the peripheral nerve and not to assess the rest of the motor unit. The motor unit is the elementary unit of neuromuscular function and any deficiency in one part of the unit may have a considerable effect on the function of the unit as a whole. Hence it is the aim of this study to assess the effect of different methods of injury and repair on the fate of motoneurones in the spinal cord and the morphological appearance and cytochemical architecture of the target muscle.

THE FATE OF MOTONEURONES IN THE SPINAL CORD AFTER PERIPHERAL NERVE REPAIR:

A QUANTITATIVE STUDY USING THE NEURAL TRACER HORSERADISH PEROXIDASE

INTRODUCTION

Skeletal muscle fibres are innervated by motor neurones which generally have their cell bodies situated in the grey matter of the spinal cord. The cell body together with the motor axon derived from it and the many muscle fibres uniquely innervated by it, is known as the motor unit. The motor unit is the elementary unit of neuromuscular function (Slater and Harris 1988).

2.1 The motoneurone pool.

The motoneurones innervating a single muscle are collectively called its motoneurone pool. Alpha (α) and gamma (γ) motoneurones are intermingled within a single motoneurone pool (Bryan et al 1972, , Burke et al 1977, Hongchien et al 1980, Peyronnard et al 1986) and are distinguished histologically by their differences in size and staining characteristics (Strick et al 1976, Burke et al 1977, Burke et al 1977, Peyronnard et al 1986,). α and γ motoneurones are considered in more detail in the Discussion section (chapter 5). The motoneurone pools tend to be segregated into longitudinal columns extending through two to four spinal segments (Romanes 1951). The columns of cells associated with the skeletal muscles are contained within Rexed's lamina IX (Rexed 1954). The position of these columns is variable with respect to the spinal segment but consistent with respect to the other columns (Romanes 1951, Nicolopoulas-Stournaras and Iles 1983). The variation in position with respect to the spinal segment is clearly illustrated by the interspecies variation seen in the position of the motoneurone

pool associated with the extensor digitorum longus (EDL), the muscle under test in the current study. It is located in lumbar segments 6 and 7 (L6 and L7) in the cat (Burke et al 1977), L3 and L4 in the rat (Peyronnard et al 1986, Nicolopoulas-Stournaras and Iles 1983, Gilmour et al 1994) and in L5 in the human (Sharrard 1955). The position of the motoneurone pool within the spinal cord, and more specifically the mediolateral position, is thought to be related to the embryonic origin of the muscle (Hollyday 1980, Landmasser 1978a). The motoneurones in the ventral horn can be distinguished in two distinct groups, one in the medial part of the ventral horn and a second much larger group which lies more laterally. The motoneurones supplying limb muscles are located laterally whilst those supplying the axial muscles are located medially. Within the lateral group the motoneurone pools are further divided into medial and lateral locations. It is the more laterally located motoneurones of the lateral group which innervate the distal muscles of the extremities and digits (Ghez 1985). The cell bodies innervating the extensor muscles tend to be located ventral to those innervating the flexors (Ghez 1985). Hence the motoneurone pool associated with EDL in the rat is located in the dorso-lateral region of the lateral group of lamina IX in the ventral horn of the L3 to L4 region of the spinal cord.

The number of motoneurones quoted as innervating any given muscle varies considerably among authors. The number of motoneurones associated with the EDL in the rat was found to be 81 by Nicolopoulas-Stournaras and Iles (1983) and 89 by Gilmour et al (1994) whereas Peyronnard et al (1986) quoted a figure of 142. Similarly the figures quoted for motoneurones associated with the soleus

muscle in the cat varied from 99 (Romanes 1951) to 270 (Boyd and Davey 1968). These variations are likely to be simply a reflection of the accuracy of the different methods of assessment used and caution must be used when considering the figures. There is a similar disagreement in the relative number of α and γ motoneurones present, with Peyronnard et al (1986) defining the percentage ratio of γ to α motoneurones as being 43.5% whilst Nicolopoulas-Stournaras and Iles (1983) claimed 32%. In each case this would appear to be a mean figure for all muscles tested. When considering individual muscles the ratio of γ to α varied from 85.8% in the tibialis anterior of the rat (Peyronnard and Charron 1983) to 24.6 % in the medial gastrocnemius of the cat (Burke et al 1977).

The number and position of the cells making up the motoneurone pool associated with a particular muscle is relatively consistent among animals of the same species (when consistent methods of assessment are used). If, however these animals undergo the severance and repair of a peripheral nerve then there are major changes in the morphology, number and position of the motoneurones.

2.2 Response of the nerve cell body to neuronal damage.

The response of the nerve cell body to neuronal damage has been described in chapter 1.

2.3 Response of the motoneurone pool to neuronal damage.

The changes seen in the motoneurone pool after the injury and repair of a peripheral nerve involve not only a decrease in the number of motoneurones present (Lieberman 1971, Carlson et al 1979, Brushart and Mesulam 1980,

McHanwell and Biscoe 1981, Brushart et al 1983, Schmalbruch 1984, Peyronnard et al 1986a, Peyronnard et al 1986b, Brushart 1990, O'Hanlon and Lowrie 1991) but also a change in the position of the motoneurone pool within the spinal cord (Brushart and Mesulam 1980, Brushart et al 1981, Brown and Hardman 1987, Brushart 1990, Mallonga et al 1991). Many authors have noted a bimodal frequency distribution of motoneurone size (Strick et al 1976 and 1977, Burke et al 1977, Nicolopoulos-Stournaras and Iles 1983, Peyronnard and Charron 1983, Peyronnard et al 1986). The cell bodies with a diameter of less than 35μm correspond to the γ motoneurones whilst the larger cells (diameter 35 - 80 μm) correspond to the α motoneurones. After the injury and repair of a peripheral nerve there is often a change in the relative number of the different cell types present (Kuno et al 1974, Brushart and Mesulam 1980, Nicolopoulos and Iles 1983, Peyronnard et al 1986a).

The position of the motoneurone pool associated with a particular muscle, in a normal animal, is variable with respect to the spinal segment but consistent with respect to the position of other motoneurone pools (Romanes 1951, Nicolopoulas-Stournaras and Iles 1983). However after the injury and repair of a peripheral nerve the peak concentration of motoneurones associated with a single muscle is regularly found to have shifted by one or even two spinal segments (Brushart and Mesulam 1980). This results in motoneurones labelled by the injection of a tracer substance into one muscle appearing within the area normally occupied by the motoneurones supplying another muscle (Brushart and Mesulam 1980, Brushart et al 1981, Brown and Hardman 1987, Brushart 1990). Thus cells which

previously innervated one muscle may reinnervate another. Indeed, Brushart (1990) showed that after the transection and repair of the sciatic nerve in the rat, an average of 53% of the motoneurones reinnervating the peroneal muscles had previously served the antagonistic tibial muscles. Such misdirected regeneration is thought to arise from the misalignment of proximal and distal axons at the suture line. The findings of Brushart (1990) that 53% of reinnervating axons are outwith the expected area after nerve section and repair and of Brown and Hardman (1987) that only 3% are outwith the expected area after a crush injury to the same nerve, fit in with this explanation. These findings imply there is an absence of neurotropism in peripheral nerve reinnervation of adult mammals (Weiss and Hoag 1946, Bernstein and Guth 1961, Miledi and Stefani 1969, Kimura et al 1975, Brushart and Mesulam 1980)

The fact that neurones may die and disappear after damage to their axons, and that the loss is particularly marked in young animals, was known before chromatolysis had been described (Gudden 1870). Mature motoneurones respond to axonal lesions with reversible chromatolysis whereas immature motoneurones degenerate more rapidly and without chromatolysis (Lieberman 1974). The difference in the degree of cell loss between new-born and older animals is illustrated by the results of Schmalbruch (1984). The sciatic nerve was sectioned in new-born, 1 week old and 4 week old rats, left for a period of 3-4 months and the associated motoneurone pool then labelled with horseradish peroxidase (HRP). After the severance of the nerve at birth there was a loss of 99% of the motoneurone pool, the loss was marked but less extreme in the animals injured at

age 1 week and the animals injured at the age of 4 weeks showed little or no difference from the motoneurone pool of normal animals. Scarisbrick et al (1992) carried out a similar study but rather than leaving the nerves severed after transection they were repaired (either direct reunion or cross reunion with inappropriate distal nerve stump). Axotomy performed on 5 and 10 day old rats resulted in significantly fewer HRP labelled motoneurones than after a similar injury in the adult. The results were consistent irrespective of whether or not the target was appropriate. There is still much debate as to the reason for the differing reaction of mature and immature neurones to axonal injury. It has been suggested that the immature neurones have underdeveloped cellular machinery involved in the response to axotomy, particularly in respect of the system synthesizing cytoplasmic proteins (Lieberman 1974). This may result in the inability of immature neurones to withstand transection of their axons. It is also possible that target organ deprivation may influence the survival of immature neurones as they control naturally occurring motoneurone death during development (Prestige 1970, Chu Whang and Oppenheim 1978a, Oppenheim 1981, Cunningham 1982).

Many authors have noted that in mature animals there is little or no difference in the motoneurone pool after the severance of peripheral nerves (Carlson et al 1979, Schmalbruch 1984). Whilst the immature animals undoubtedly experience a greater degree of cell loss, the claim that mature animals show little or no difference in the motoneurone pool after the severance of their peripheral nerves is in direct contrast to many other studies (Lieberman 1974, Brushart and Mesulam 1980, Brushart et al 1983, Peyronnard et al 1986, Gilmour et al 1994 in

press). These studies all noted a significant decrease in the number of motoneurones present after injury compared to normal. The degree of cell loss produced by peripheral neurotomy is dependent on a variety of factors such as the species of animal, the nature of the injury, the level of the injury and whether the injury to the nerve had been repaired, left alone or prevented from regenerating. The more severe the lesion the greater the degree of cell loss, hence neurotmesis produces more cell loss than crush injuries and resection or avulsion of the nerve a greater degree still (Lieberman 1974, Peyronnard et al 1988). Lesions nearer to the cell body produce a greater degree of cell loss than lesions which occur more distally (Geist 1933, Barr and Hamilton 1948, Kline et al 1964a, Carlson et al 1979, Mackinnon et al 1985, Ygge 1989). The degree of cell loss is much less marked where the nerve has been allowed to regenerate and reform functional connections (Weiss, Eds and Cavanaugh 1945). There has been a very limited number of studies on the effect of the method of repair on the survival of cells within the motoneurone pool and the results of these two studies directly contradict one another (Brushart et al 1983, Brushart 1991).

It is crucial for us to know the fate of spinal motoneurones after the surgical repair of peripheral nerves, since the extent of the recovery of function is presumed to be dependent on the anatomical injury. It would be futile to repair a peripheral nerve if the surgical technique employed had a deleterious effect on the motoneurone pool, as this would reduce rather than enhance recovery. It is the aim of this study to assess the changes which occur in the motoneurone pool following the repair of a specific peripheral nerve using several clinically appropriate surgical

techniques. To date no such long term comparative study has been undertaken.

2.4 Horseradish peroxidase - a neural tracer.

The neural tracer horseradish peroxidase has been used in the present study to assess the changes in the motoneurone pool associated with EDL in the rat. HRP neurohistochemistry has become one of the most frequently used methods for tracing neural connections within the central nervous system (Mesulam 1978). Peroxidase activity was first discovered in 1855, by Schoenbeun, however it was almost a century later before it was isolated, purified and crystallized. Even then it was not until the 1960's that a definitive demonstration of a neural connection was obtained using this technique. The introduction of HRP revolutionized neuroanatomy, by providing a reliable means of tracing the connections of the CNS, peripheral ganglia, nerves, muscle and skin (Mesulam 1978).

A variety of methods can be used to administer HRP, from the direct application of crystals onto the severed nerve, to the use of slow release gels or the injection of the an aqueous solution directly into the target muscle. The advantages and disadvantages of these methods will be discussed later. Once administered, the HRP gains entry into the neurones through a process of endocytosis at the neuromuscular junctions and sensory nerve terminals (Hongchien et al 1980, Mesulam 1982). Endocytotic vesicles which contain the enzyme are then transported retrogradely along the neural processes which spread from the site of administration (Mesulam 1982). The vesicles subsequently accumulate in the cell bodies of the neurones in the ventral horn of the spinal cord.

The HRP molecules are not visible, however they do have a readily detectable reaction product which can be obtained by means of an enzymatic reaction. The reaction involves the tissue bound HRP combining with its substrate hydrogen peroxide to produce an [HRP H_2O_2] complex. The resultant complex can oxidize a wide variety of chromagen hydrogen donors (Mesulam 1982). The oxidized chromagens assume a dense colour and precipitate out as readily detectable markers of HRP activity, i.e.

$$HRP + H_2O_2 \rightarrow [HRP.\ H_2O_2]$$

$$[HRP.\ H_2O_2] + (CHR)\ H_2 \rightarrow (CHR) + HRP + 2\ H_2O$$

Thus neural pathways can readily be traced.

Despite the fact that HRP neurohistochemistry is one of the most widely used methods for tracing neural connections, there is still a great deal of debate about its accuracy and reliability. Consequently, a great deal of research has been carried out to assess the degree of accuracy and reliability that can reasonably be expected from the use of HRP as a neural tracer. These studies have assessed the number of motoneurones labelled, preferential labelling of α-motoneurones, alterations in the transport of HRP in injured neurones, the method of administration of HRP and the sensitivity of different enzymatic reactions. Each of these problems has been considered and is discussed in detail in chapter 5.6.

2.5 Aims of the study.

This study aims to answer the following questions:-

1. What changes occur in the number of motoneurones associated with a

specific muscle after the injury and repair of the peripheral nerve which supplies it?

2. What changes occur in the distribution of motoneurones associated with a specific muscle after the injury and repair of the peripheral nerve which supplies it?

3. Does the technical method of repair have a specific effect upon events occurring in the motoneurone pool?

Materials and Methods

3.1 Surgical procedures

The experimental groups each consisted of 5 male Sprague Dawley rats, of 200-300 grams initial weight. Prior to the operation general anaesthesia was induced by means of an intramuscular injection of 1:1 mix of Hypnorm (Jansen Pharmaceuticals, UK, 0.5ml kg^{-1}) and Hypnovel (Midazolam Hydrochloride 5mg ml^{-1}, Roche Products Ltd., Welwyn Garden City). In each of the rats, an incision was made over the iliac crest in order expose biceps femoris. The muscle was then detached from its ilial origin and deflected distally to expose the left sciatic nerve. Once the nerve had been freed from the surrounding connective tissue, it was transected and repaired using one of the following techniques:

(a) Freeze-thawed coaxial skeletal Muscle Graft

A section of biceps femoris (not less than 2cm x 1cm) was excised, ensuring that the long axis lay parallel to the direction of the muscle fibres. The piece of muscle was pinned out on a square of card, ensuring that no tension was applied, and then placed in liquid nitrogen until thermal equilibrium was established. The muscle block was then transferred to sterile distilled water where it was allowed to thaw - a process which took approximately 5 minutes. Once thawed, the muscle block was trimmed to form a rectangular graft of approximately 1cm in length by 2mm in diameter. Particular attention was paid to the formation of the graft, to ensure that the muscle fibres ran parallel to the long axis of the graft. The graft was then introduced into the gap in the nerve and secured by means of 4-6 10/0

interrupted sutures (Ethilon polyamide, Ethicon UK Ltd.) at the proximal and distal stumps (Figure 3.1.1). The remains of biceps femoris were repositioned over the repaired nerve, being stretched and then secured to the lumbar fascia by means of 6/0 interrupted sutures (covered Vicryl, Ethicon UK Ltd.). 3/0 interrupted sutures (covered Vicryl, Ethicon UK Ltd.) were used for skin closure. The rats were then returned to normal animal house conditions.

(b) Nerve Graft

A 1cm. section of the exposed sciatic nerve was excised then reintroduced into the gap in the nerve and secured by means of 4-6 10/0 interrupted sutures (Ethilon polyamide, Ethicon UK Ltd.) at the proximal and distal stumps (Figure 3.1.2). The repair was then covered and the skin closed as before.

(c) Direct Epineurial Suture

The exposed sciatic nerve was transected and then sutured back into position using 4-6 10/0 interrupted sutures (Ethilon polyamide, Ethicon UK Ltd.) - Figure 3.1.3. The repair was then covered and the skin closed as before.

(d) Crush Injury

The exposed sciatic nerve was placed in a pair of smooth jawed micro-needle holders which were closed to the second ratchet and held for 10 seconds. On release, the crushed area of the nerve was found to be opaque in appearance (Figure 3.1.4). No formal repair was implemented. The injured area of nerve was covered and the skin closed as before.

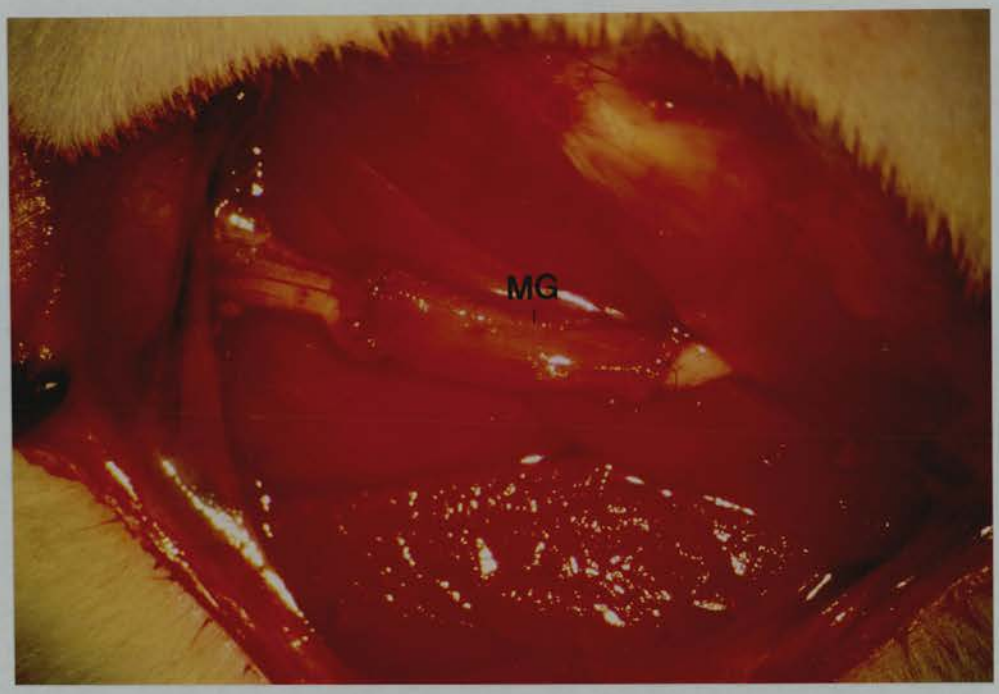

Figure 3.1.1 - Transected sciatic nerve repaired with a 1cm freeze thawed coaxially aligned skeletal muscle autograft (MG).

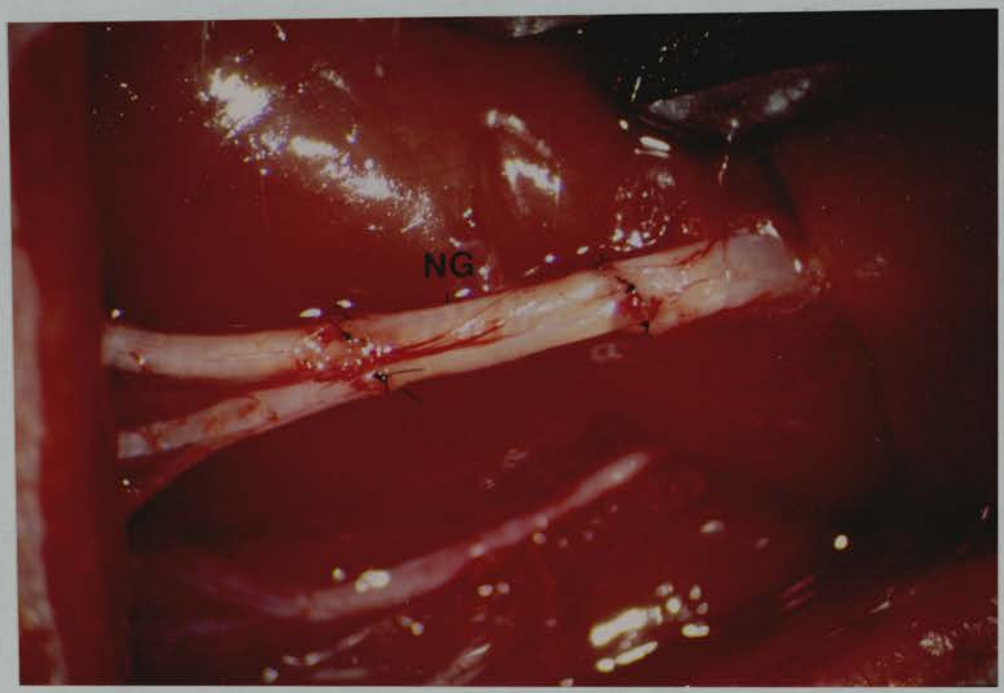

Figure 3.1.2 - Transected sciatic nerve repaired with a 1cm full thickness nerve autograft (NG).

Figure 3.1.3 - Transected sciatic nerve repaired by a direct epineurial suture (S = suture line between proximal and distal stumps).

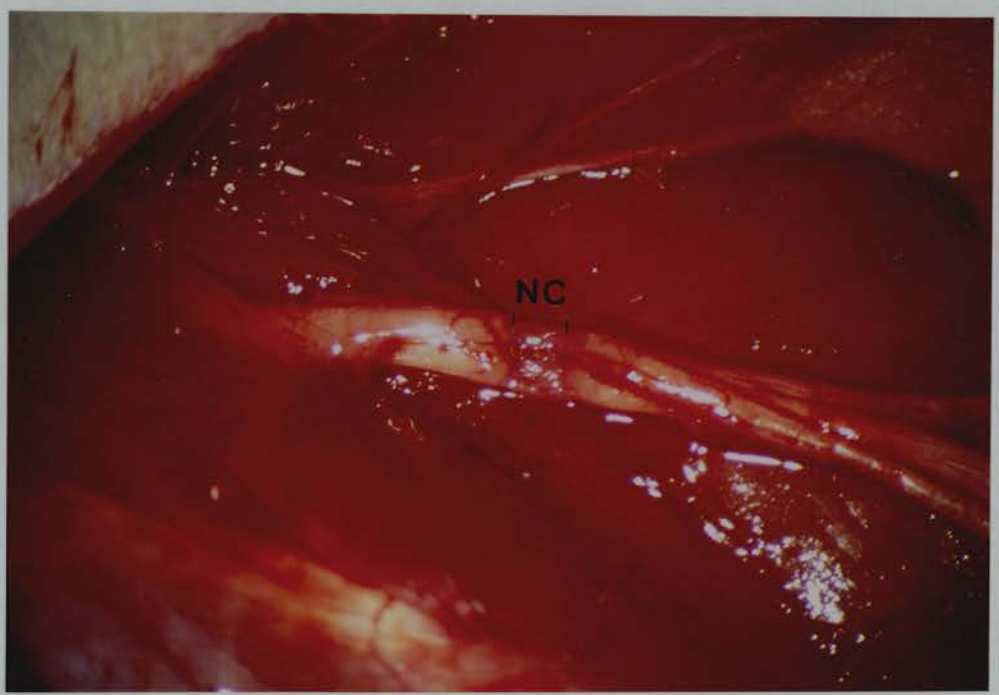

Figure 3.1.4 - Sciatic nerve after standardized nerve crush injury (NC). Note the opaque appearance of the crushed area of the nerve.

As should be the case in the surgical repair of any peripheral nerve particular attention was paid to accuracy during suturing. Every effort was made to ensure that the nerve was not twisted or put under tension during repair. With the use of a Weck Fibermatic (Q902A1) operating microscope it was possible to recognize landmarks such as longitudinal epineurial blood vessels and the fascicular pattern of the cut ends of the nerve which were used to ensure correct orientation of the stumps before suturing. The sutures were positioned in such a way that they only passed through the extreme periphery of the graft and/or epineureum of the nerve thus ensuring they did not cause further damage to the nerve, further disruption of the blood supply or obstruct the path of the regenerating nerves. Care was also taken to ensure that the sutures were tight enough to be secure but not so tight as to cause distortion or tension at the suture line

The motoneurone pool was assessed at 50, 100, 200 and 300 days after operation using the neural tracer horseradish peroxidase (HRP).

3.2 Administration of HRP

The rats were anaesthetized by means of an intramuscular injection of a 1:1 mix of Hypnorm (Jansen Pharmaceuticals, UK, 0.5ml kg^{-1}) and Hypnovel (Midazolam Hydrochloride 5mg ml^{-1}, Roche Products Ltd., Welwyn Garden City, UK). In each of the rats, an incision was made over the iliac crest in order to expose biceps femoris. The muscle was detached from its iliac origin and deflected distally, thus exposing the transected and repaired sciatic nerve. The nerve was traced distally to its point of bifurcation, from where each of the main branches was individually traced and freed from the surrounding connective tissue (Figure 3.2.1).

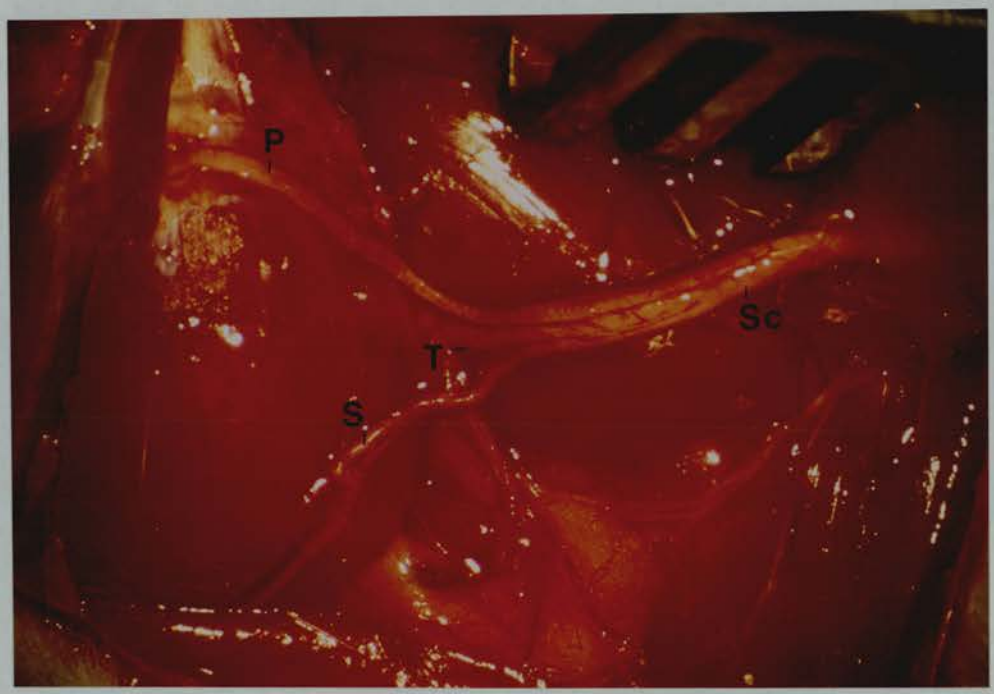

Figure 3.2.1 - Dissection of the sciatic nerve (Sc) showing the peroneal (P), tibial (T) and sural (S) branches.

The tibial and sural nerves were transected and cauterized, in order to ensure that the only possible route from the extensor compartment of the lower limb to the spinal cord was the EDL branch of the peroneal branch of the repaired sciatic nerve. Thus there could be no unwanted uptake of HRP from the surrounding tissues. The incision was then extended distally, along the full length of the tibia, thus exposing tibialis anterior. This muscle was detached from its tibial origin and deflected proximally, in order to expose the EDL. The needle of a Hamilton microsyringe was inserted into the EDL and 50 µl. of 20% HRP solution (Sigma Chemicals, UK, Ltd.), in normal saline, was injected along the length of the muscle. Great care was taken to ensure that all areas of the muscle underwent a colour change as this was taken to imply adequate and complete infiltration of the HRP solution (Figures 3.2.2 and 3.2.3).

The rats were returned to normal animal house conditions for 48-72 hours, in order to allow the transport and accumulation of HRP in the spinal motoneurones.

3.3 Tissue Fixation

A transcardial perfusion was performed, using procedure II of Rosene and Mesulam (1978). The rats were anaesthetized as before and the chest opened by a median stenotomy. A vasodilator (1 ml 1% sodium nitrite) was injected into the left ventricle, rapidly followed by a 50 ml physiological saline flush (at room temperature) and 500 ml of 1.25% glutaraldehyde and 1% paraformaldehyde in 0.1 M phosphate buffer (pH 7.4 at room temperature). The fixation process was immediately followed by a flush with 100 ml 10% sucrose buffer solution at 4°C.

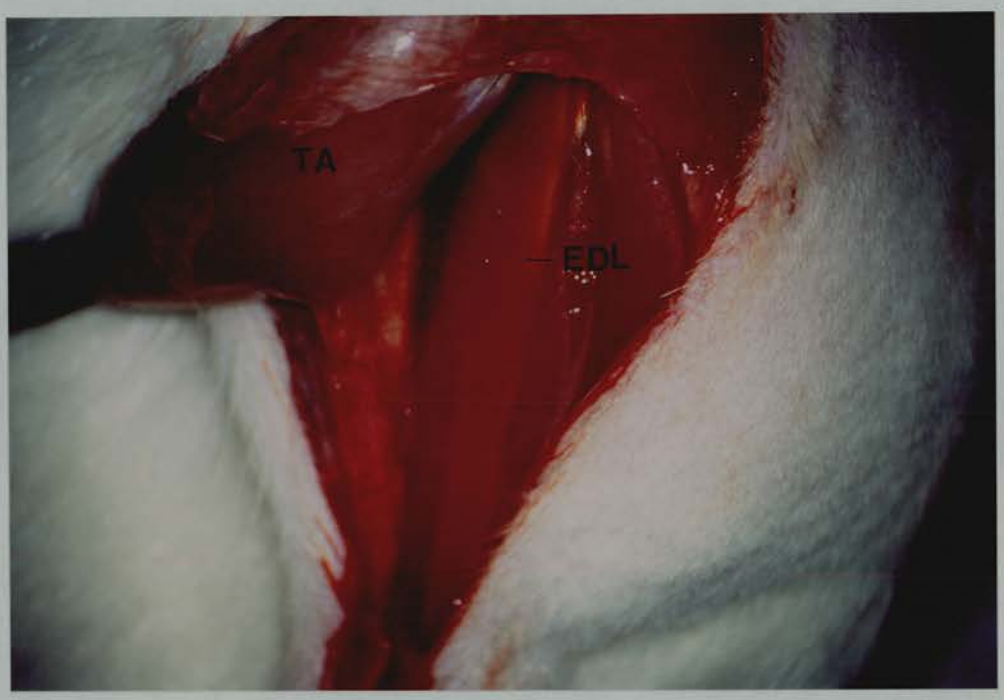

Figure 3.2.2 - Extensor digitorum longus (EDL) before the injection of the horseradish peroxidase solution - note the bright red colour of the muscle. T.A.= deflected tibialis anterior.

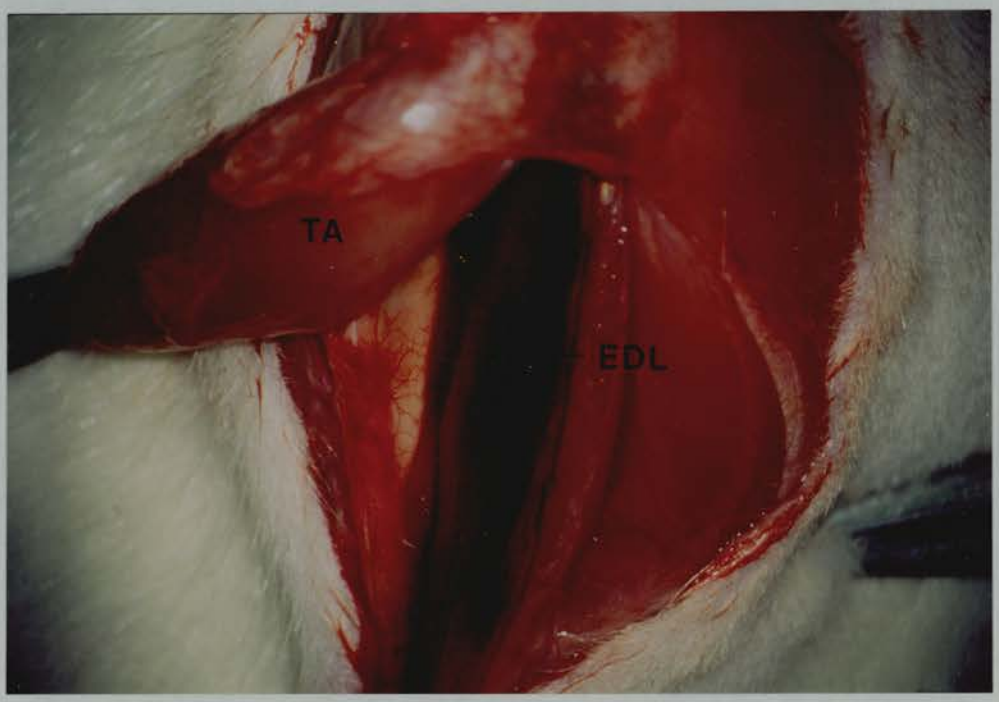

Figure 3.2.3 - Extensor digitorum longus (EDL) after the injection of the horseradish peroxidase solution - note the colour change of the muscle from bright red to dark brown. T.A.= deflected tibialis anterior.

3.4 Tissue Sectioning

When adequate fixation of the tissue had been achieved, the spinal cord was exposed through a long posterior laminectomy and a 1.5 cm portion excised from the L3 to L5 region. The excised tissue was placed in fresh sucrose buffer solution and stored at 4°C for a minimum of 3 hours, more usually 12-24 hours, prior to sectioning. The tissue was embedded in Tissue Freezing Medium (Reichert-Jung, Cambridge Instruments) and frozen using a Freeze Jet aerosol (Agar Scientific Ltd., Essex.). A Frigocut 2800E Cryostat (Reichert-Jung, Cambridge Instruments) was used to cut 50 um serial sections, at -20 to -25°C. The serial sections were floated out in phosphate buffered saline (PBS) pH 7.4, where they could be stored for up to 7 days prior to the enzymatic reaction.

3.5 Enzymatic Reaction

The sections underwent an enzymatic reaction, based on the tetramethyl benzidine (TMB) procedure as described by Mesulam (1978). The sections were rinsed in several changes of distilled water before to a 20 minute pre-reaction soak in the TMB solution. The addition of 5ml of 0.3% hydrogen peroxide to the TMB solution formed the incubation solution for the enzymatic reaction. The recommended incubation period of 15 minutes was found to be excessive, in practice the colour change of the incubation solution provided an accurate guide to appropriate timing. The details of this method are explained more fully in "Steps taken to avoid systematic errors". After the enzymatic reaction the sections were rinsed in several changes of distilled water, mounted onto chrome alum coated slides and allowed to dry overnight. The sections were counterstained for 2.5

minutes with 1% neutral red, dehydrated, cleared and mounted. All sections and slides were stored at 4°C, since enzyme denaturation is greatly reduced at lower temperatures.

Details of all solutions can be found in Appendix 1.

3.6 Controls

Five untreated rats of the same age were used as normal controls for comparison. Additionally, a further six rats were used as controls in order to assess possible HRP leakage. The six animals were injected with HRP as described previously. In three animals the EDL was denervated by cutting all supplying branches from the peroneal nerve and in the other three the peroneal nerve was ligated with 3/0 silk at the knee. The tibial and sural nerves were then transected and cauterized as before. No reaction product was subsequently found in the motoneurones of the spinal cord. The lack of reaction product indicated that there was no significant leakage of HRP from the EDL to the surrounding tissue and that denervation of the tibial and sural territories had been complete.

3.7 Analysis of labelled motoneurones in the spinal cord.

The sections were examined microscopically and a Magiscan morphometric analysis system (Joyce-Loebl, UK Ltd.) used to obtain uncorrected counts of the motoneurones associated with the EDL. The motoneurone pool associated with the EDL could readily be identified, within the ventral horn of the spinal cord, by the HRP labelling which gave the cells a dark blue granular appearance (Figures 3.7.1 and 3.7.2). A correction factor for split cells was applied (Abercrombie 1946), taking into account the size of the cell and the thickness of the section.

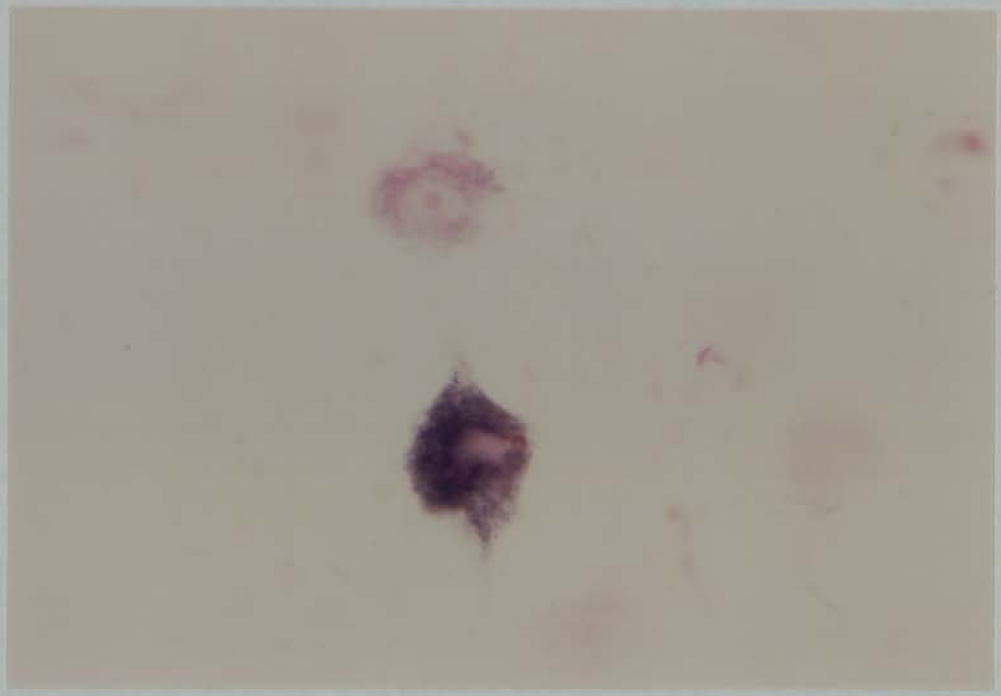

Figure 3.7.1 - Horseradish peroxidase (HRP) labelled motoneurones in the anterior horn of the L4 region of the spinal cord. Note the dark blue granules of HRP within the cell body.

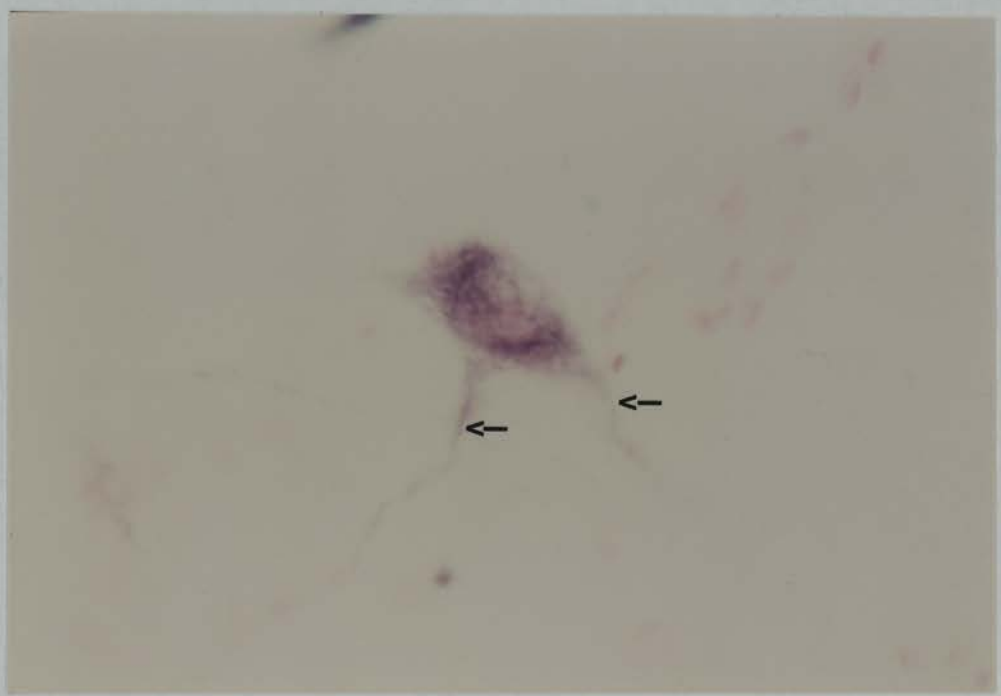

Figure 3.7.2 - HRP labelled motoneurones in the anterior horn of the L4 region of the spinal cord. Arrows indicate HRP labelling of the dendrites.

Details of the correction factor are given in appendix 2.

All data processing and statistical analysis was carried out using Microsoft Excel version 5.

3.8 Statistical Analysis

The null hypothesis.

In the statistical analysis of data one must first describe a null hypothesis. A null hypothesis assumes that there is no real difference between different samples and statistical analysis aims to prove or disprove the null hypothesis. In the current study the null hypothesis states that there is no real difference in the number and size of spinal motoneurones associated with a particular muscle after each type of peripheral nerve injury and repair.

The normal distribution.

The normal distribution is represented by a family of curves which express a certain relationship between the mean and the variance (the square of the standard deviation). The curve of a normal distribution is symmetrical about the mean and is bell shaped, however the shape (height and width) of the bell is dependent on the standard deviation. It is important to test the distribution of all data prior to statistical analysis as only normally distributed data can be analyzed using parametric tests. If data was not normally distributed then non-parametric tests, which are less sensitive, must be used. In the current study, the distribution of data was tested for normality by plotting frequency histograms and assessing the skewness of the curve.

Analyses of variance (ANOVA).

ANOVA was used to analyze the data. The advantage of using ANOVA is that all the data were pooled and hence a better estimate of the error variances was obtained. Additionally, since the data were pooled, the one calculation analyzed all the samples simultaneously. ANOVA was used to analyze whether the mean number of labelled cells seen after each of the methods of injury and repair could be drawn from populations with the same mean. The ANOVA was calculated for each of the repair methods (collectively) and for each of the time periods (collectively). The results indicated that there was a significant difference in the variance for each repair type, hence Student's t-tests were carried out to assess which results were significantly different from one another.

The Student's t-test.

The two sample students t-test was used to assess the whether the difference in results between the different repair types and time periods reached a significant level, either when compared to one another or to control values.

Mann-Whitney U test.

The Mann-Whitney U test was used where a normal distribution could not be shown.

Statistical significance.

The statistical significance was assessed at the 5% level. If there is more than a 5% probability that the difference between two samples has occurred by chance then the null hypothesis is accepted, i.e. there is no significant difference between the samples. If there is less than a 5% probability that the difference between two

samples has occurred by chance then the null hypothesis is rejected, i.e. the samples are significantly different. The results are summarized in tables 4.2.2, 4.2.3, 4.3.2 and 4.3.3.

3.9 Steps taken to avoid systematic errors

The variation between animals was kept to a minimum by ensuring that animal species, sex and weight were consistent in the groups. The method of choice for the administration of the neural tracer in the current study was intramuscular injection. The reasons for the use of this method and the alternatives are discussed later - see Chapter 5.6. The needle of a Hamilton microsyringe was inserted along the length of the muscle and the HRP solution injected as the needle was slowly withdrawn. Injecting in this way increased the likelihood that all areas of the muscle would be infiltrated by the solution. The muscles were examined after injection to ensure that all areas had undergone a colour change as this was taken to imply adequate and complete infiltration of the HRP solution. Subsequent dispersal of the solution to all parts of the muscle was aided by fasciculation of the muscle that was seen consistently after the injection. If such efforts were not made to ensure the complete infiltration of the muscle, it is likely that errors would have been introduced as the entire motoneurone pool would not subsequently have been labelled and therefore assessed.

After the injection of the HRP solution the animals were returned to normal animal house conditions for 48 - 72 hours. A pilot study was undertaken to assess the optimum time period which should be allowed for the transport and accumulation of HRP in the spinal motoneurones. It was found that results from

animals left for less than 24 hours or greater than 72 hours were very variable. This was most likely to be because of insufficient time for the transport and accumulation of HRP in animals left for the shorter time period and to the denaturation of the enzyme after the longer time period. In agreement with Mesulam (1978), it was found that killing and assessing the animal any time between 48 and 72 hours gave consistent and reproducible results.

Every effort was made to ensure that the animals were adequately perfused to ensure satisfactory fixation of the tissues. Despite being consistent in the method used for the perfusion, the results, in terms of fixation, were variable. It is virtually impossible to totally standardize the perfusion technique which is influenced by, among other things, the properties of the animal's vascular system. In cases where fixation was felt to be incomplete, the required tissues were further fixed in 1.25% glutaraldehyde and 1% paraformaldehyde in 0.1 M phosphate buffer (at 4°C, pH 7.4). The timing of such additional fixation was carefully monitored since, although inadequate fixation is undesirable, over exposure to unbound aldehydes results in the depression of enzyme activity. Either eventuality may introduce errors in the subsequent assessment of the motoneurone pool.

The enzymatic reaction, which is notoriously risky, was based on the TMB method of Mesulam (1978). The reasons for the use of this method and the alternatives are discussed later - see chapter 5.6. The chemicals used in the reaction were always obtained from the same supplier and were replaced regularly to ensure their quality and purity. The recommended incubation period of 15 minutes was found to be excessive, in practice the colour change of the incubation solution provided an accurate guide to appropriate timing. The solution gradually changes

colour from pale brown to green to dark blue. The sections were removed when the solution was on the brink of turning blue (the "tide mark" around the staining dish provided an excellent indication!). The use of a colour change in the solution to indicate appropriate timing of incubation is subjective and thus represents an obvious source of error. This error was eliminated, as far as possible, since all reactions were carried out personally.

All sections, whether free-floating or slide-mounted, were stored at 4°C since enzyme denaturation is greatly reduced at lower temperatures.

A count was made of all HRP labelled cell bodies in the ventral horn of the spinal cord. An obvious source of error, which may greatly exaggerate the number of labelled cells counted, is the presence of split cells. This error was minimized by applying the correction factor of Abercrombie (1946) which is described in detail in Appendix 2. The reasons for the use of this method and the alternatives are discussed later - see chapter 5.7. A similar exaggeration in the number of labelled cells may occur if the HRP solution leaks out of the target muscle and into the surrounding tissues. This would obviously result in the uptake of HRP from the surrounding tissues and the subsequent labelling of motoneurone pools not associated with the target muscle. This possible source of error was assessed and eliminated in a series of control experiments - see chapter 3.6 "Controls".

RESULTS

All rats showed progressive signs of recovery up to 300 days after operation, by which time the toe-spreading reflex could be elicited in 92% of experimental animals. The return of this reflex is said to reflect good motor and sensory reinnervation by the sciatic nerve (Myles et al 1992). The 8% of animals who failed to regain their toe-spreading reflex on the repaired side were found to have acquired fixed deformities of the ankle joint and this prevented any movement of the foot. This occurred in 5 animals whose nerves had been transected and then repaired by means of the muscle graft and in one which received a nerve graft.

4.1 Postoperative changes in the distribution of spinal motoneurones.

The labelled motoneurone pool associated with EDL was located within the L3, L4 & L5 levels of the spinal cord. In control animals the cells formed a discrete population, concentrated primarily in lower L4 and L5, whereas in experimental animals the labelled cells were more widely distributed and extended from upper L3, through L4 to L5.

4.2 Postoperative changes in the number of spinal motoneurones.

Table 4.2.1 shows the change in the mean number of labelled motoneurones in the spinal cord after each method of injury and repair and at each time period after operation. Tables 4.2.2 and 4.2.3 show whether the difference in the number of labelled cells found after each of the methods of injury and repair and at each time period after operation is significantly different, either when compared to one another or to control values. Significance was assessed at the 5% level.

Repair	Mean no. of labelled motoneurones			
	50 days	100 days	200 days	300 days
Muscle Graft	39.98 ± 16.77	46.01 ± 25.60	66.36 ± 28.96	77.30 ± 22.28
N-N Suture	50.49 ± 25.75	61.55 ± 16.03	49.38 ± 18.80	66.70 ± 7.72
Nerve Graft	27.91 ± 20.55	44.24 ± 11.61	41.29 ± 17.94	61.21 ± 13.57
Nerve Crush	77.25 ± 21.35	72.39 ± 12.67	69.02 ± 15.97	69.73 ± 18.97
Control	89.82 ± 2.61	89.82 ± 2.61	89.82 ± 2.61	89.82 ± 2.61

Table 4.2.1 - The mean number (± standard deviation) of HRP labelled motoneurones at L3 to L5 level of spinal cord after each method of injury and repair and at each time period after operation.

(A)

Repair	50 Days				
	Control	Muscle Graft	Nerve Crush	Nerve Graft	N-N Suture
Control	*	*	*	*	*
Muscle Graft	P<0.01	*	*	*	*
Nerve Crush	N.S.	P<0.05	*	*	*
Nerve Graft	P<0.05	N.S.	P<0.01	*	*
N-N Suture	P<0.05	N.S.	N.S.	N.S.	*

(B)

Repair	100 Days				
	Control	Muscle Graft	Nerve Crush	Nerve Graft	N-N Suture
Control	*	*	*	*	*
Muscle Graft	P<0.01	*	*	*	*
Nerve Crush	P<0.05	P<0.05	*	*	*
Nerve Graft	P<0.01	N.S.	P<0.01	*	*
N-N Suture	P<0.01	N.S.	N.S.	N.S.	*

(C)

Repair	200 Days				
	Control	Muscle Graft	Nerve Crush	Nerve Graft	N-N Suture
Control	*	*	*	*	*
Muscle Graft	N.S.	*	*	*	*
Nerve Crush	P<0.05	N.S.	*	*	*
Nerve Graft	P<0.01	N.S.	P<0.05	*	*
N-N Suture	P<0.05	N.S.	N.S.	N.S.	*

(D)

Repair	300 Days				
	Control	Muscle Graft	Nerve Crush	Nerve Graft	N-N Suture
Control	*	*	*	*	*
Muscle Graft	N.S.	*	*	*	*
Nerve Crush	N.S.	N.S.	*	*	*
Nerve Graft	P<0.05	N.S.	N.S.	*	*
N-N Suture	P<0.01	N.S.	N.S.	N.S.	*

Table 4.2.2 - Significance values for the comparison of the mean number of HRP labelled motoneurones at L3 to L5 level of spinal cord after each method of injury and repair at (A) 50 days, (B) 100 days, (C) 200 days and (D) 300 days after operation.

(A)

Duration	Muscle Graft			
	50 days	100 days	200 days	300 days
50 days	*	*	*	*
100 days	N.S.	*	*	*
200 days	N.S.	N.S.	*	*
300 days	P<0.05	P<0.05	N.S.	*

(B)

Duration	Nerve Crush			
	50 days	100 days	200 days	300 days
50 days	*	*	*	*
100 days	N.S.	*	*	*
200 days	N.S.	N.S.	*	*
300 days	N.S.	N.S.	N.S.	*

(C)

Duration	Nerve Graft			
	50 days	100 days	200 days	300 days
50 days	*	*	*	*
100 days	N.S.	*	*	*
200 days	N.S.	N.S.	*	*
300 days	P<0.05	N.S.	N.S.	*

(D)

Duration	N-N Suture			
	50 days	100 days	200 days	300 days
50 days	*	*	*	*
100 days	N.S.	*	*	*
200 days	N.S.	N.S.	*	*
300 days	N.S.	N.S.	N.S.	*

Table 4.2.3 - Significance values for the comparison of the mean number of HRP labelled motoneurones at L3 to L5 level of spinal cord at each time period after injury and repair with a (A) muscle graft, (B) nerve crush, (C) nerve graft and (D) nerve to nerve suture.

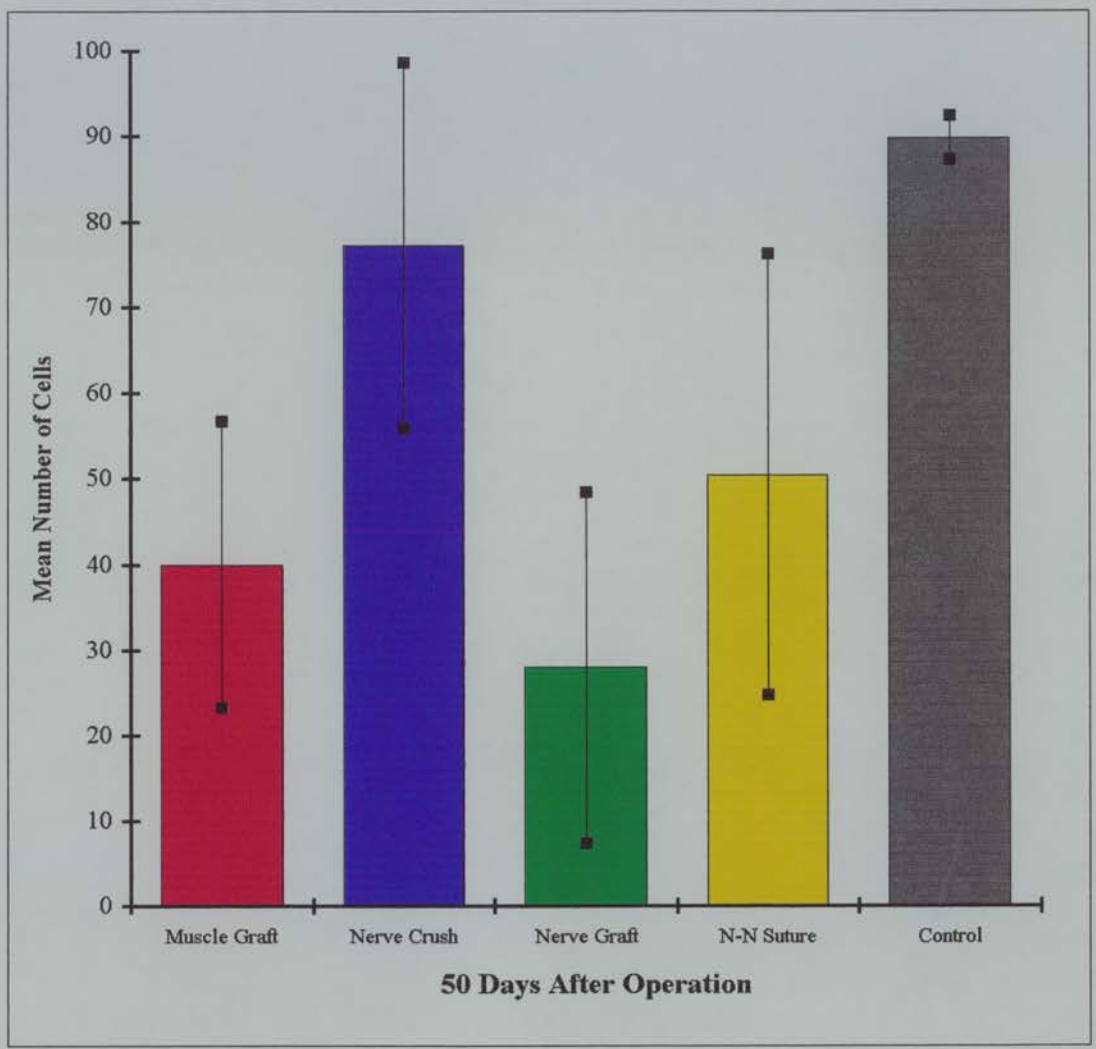

Figure 4.2.1 - Mean number (± standard deviation) of HRP labelled motoneurones at L3 to L5 level of spinal cord 50 days after operation.

Figure 4.2.1 shows the mean ± standard deviation of the mean number of labelled motoneurones in the spinal cord of control animals and in animals reviewed 50 days after injury and repair by each of the different methods. The standardized crush injury resulted in a loss of 14% of the normal cell population as compared to the significantly greater loss of 43.79%, 55.49% and 68.92% of cells following a direct epineurial suture ($p < 0.05$), freeze thawed muscle autograft ($p < 0.01$) and full thickness nerve autograft ($p < 0.05$) respectively. The numbers of labelled cells associated with the latter three groups were not significantly different from one another. The two methods of repair which involved the insertion of a graft resulted in significantly fewer labelled cells than after the less severe injury of a nerve crush - significance was achieved at the 5% level for the freeze thawed muscle autograft group and at the 1% level for the full thickness nerve autograft. 100 days after nerve injury and repair (Figure 4.2.2) the nerve crush was still associated with a greater number of labelled cells (loss of 13.64%, i.e. 70 cells). This difference was only significant when compared to the muscle graft and nerve graft results - significance was achieved at the 5% level for the freeze thawed muscle autograft group and at the 1% level for the full thickness nerve autograft. The number of cells labelled after the nerve crush injury was less than in control animals ($p < 0.05$). The three injuries involving transection of the nerve were also associated with fewer labelled motoneurones than normal animals although this was significant at the 1% level. By 200 days the trend had changed somewhat with the muscle graft associated with many more labelled cells than either the nerve graft or the direct epineurial suture and producing a very similar result to the nerve crush

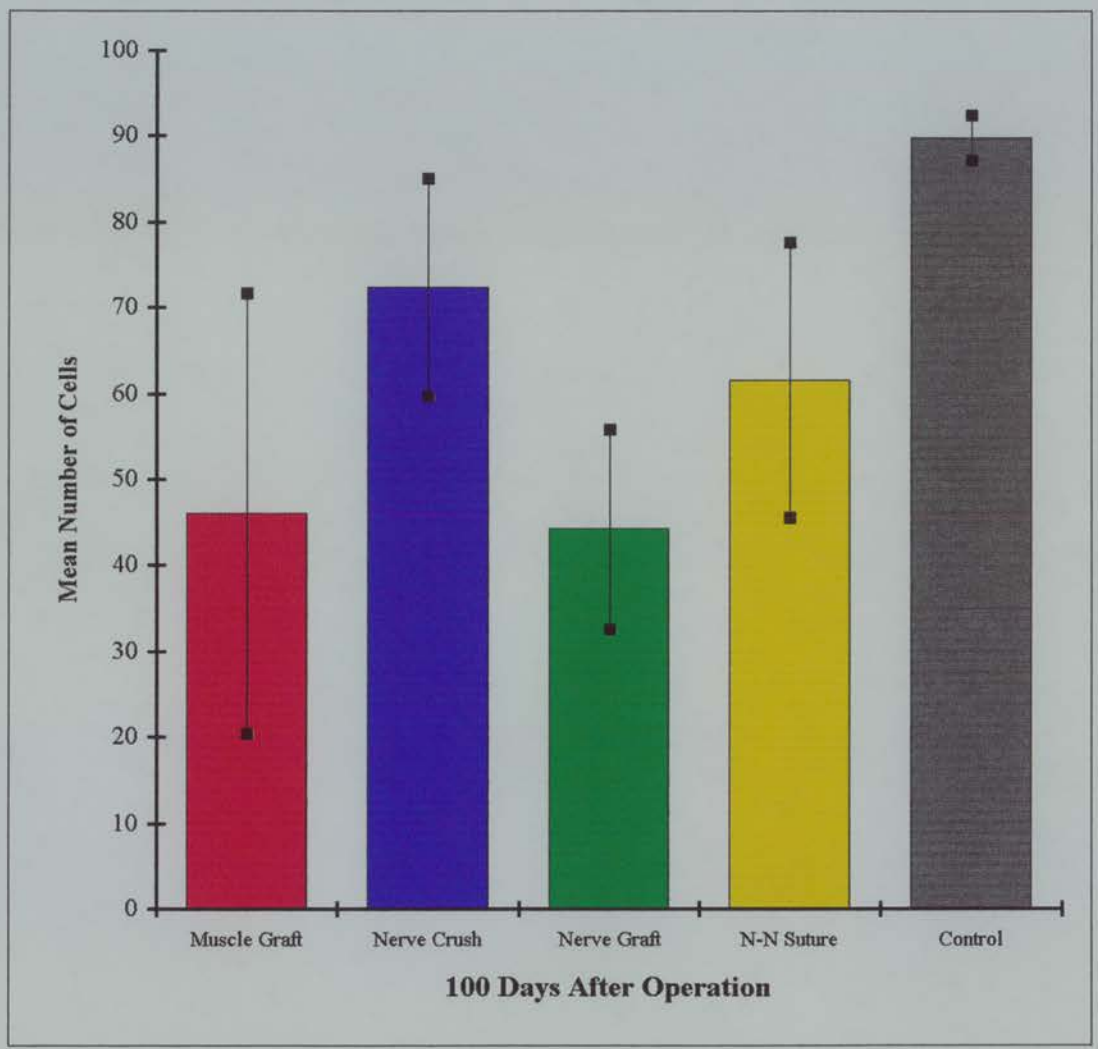

Figure 4.2.2 - Mean number (± standard deviation) of HRP labelled motoneurones at L3 to L5 level of spinal cord 100 days after operation.

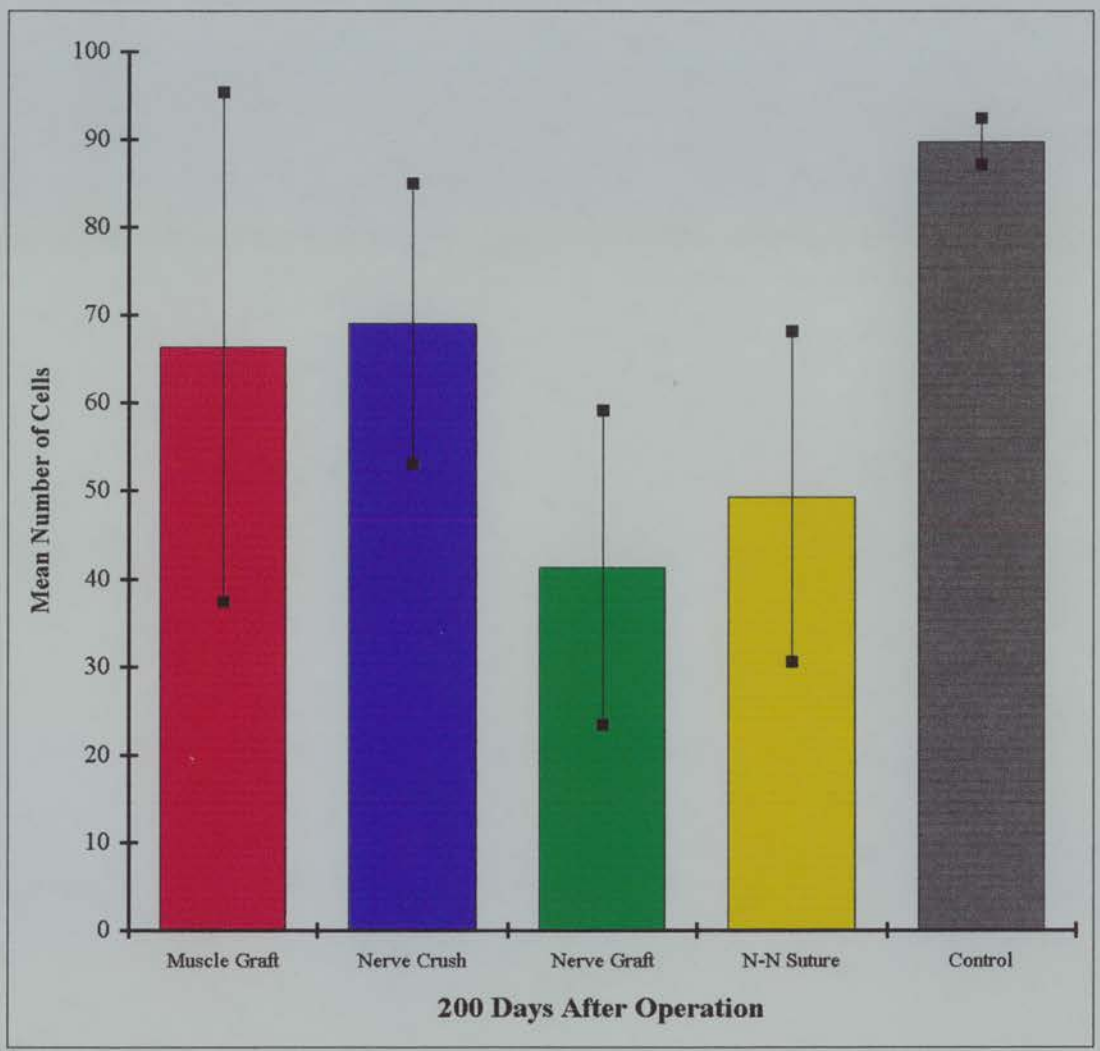

Figure 4.2.3 - Mean number (± standard deviation) of HRP labelled motoneurones at L3 to L5 level of spinal cord 200 days after operation.

(Figure 4.2.3). Indeed the results of the muscle graft group were not significantly different from control values which is in direct contrast to all other methods of injury and repair ($p < 0.05$ for nerve crush and direct epineurial suture group and $p < 0.01$ for nerve graft group). On comparing the results of the four methods of injury and repair with one another, only the results of the nerve graft and nerve crush groups were found to be significantly different at the 5% level. At 300 days after operation (Figure 4.2.4) there was no significant difference between any of the experimental groups when compared to each other, although the nerve graft ($p < 0.05$) and direct epineurial suture ($p<0.01$) results were significantly less than normal control values.

Figure 4.2.5 shows the change in the mean number of labelled motoneurones in the spinal cord after each method of injury and repair and at each time period after operation. Initially, the three groups which had undergone neurotmesis all had significantly fewer ($p < 0.01$ after muscle graft and nerve graft, $p < 0.05$ after direct epineurial suture and nerve crush injury) labelled cells than the control animals. With time, the number of labelled cells associated with the muscle graft repair increased steadily from 44.51% to 51.22%, 73.89% and 86.06% of normal values at 50, 100, 200 & 300 days respectively. The number of labelled cells 300 days after operation was significantly greater than at 50 days ($p < 0.05$). Animals treated with a full thickness nerve autograft followed a similar steady increase with time from 31.08% at 50 days to 49.26%, 45.97% and 68.15% of normal population at 300 days, although the percentage of cells labelled always remained less than that associated with the muscle graft. Again, the number of labelled cells 300 days after operation was significantly greater than at 50 days ($p < 0.05$).

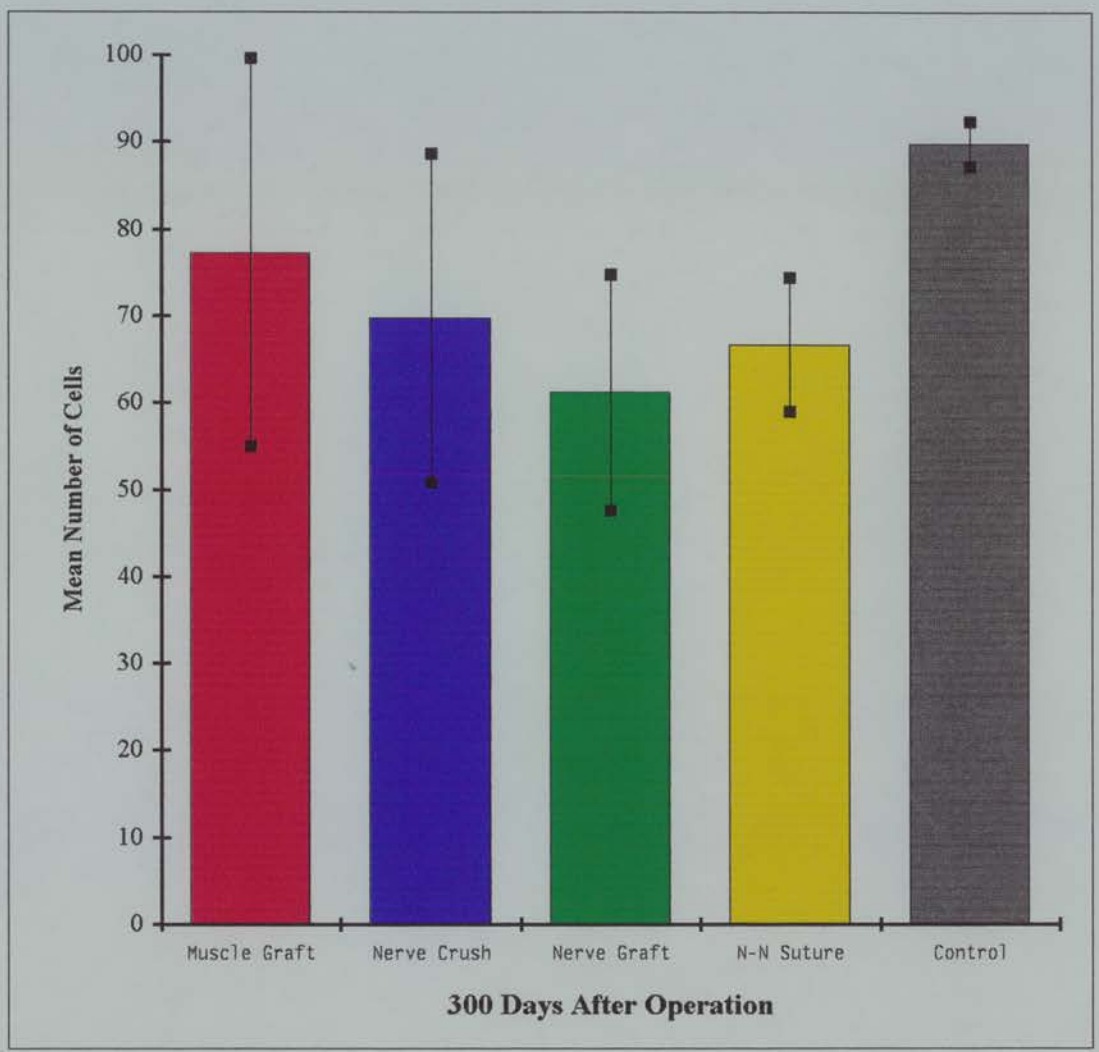

Figure 4.2.4- Mean number (± standard deviation) of HRP labelled motoneurones at L3 to L5 level of spinal cord 300 days after operation.

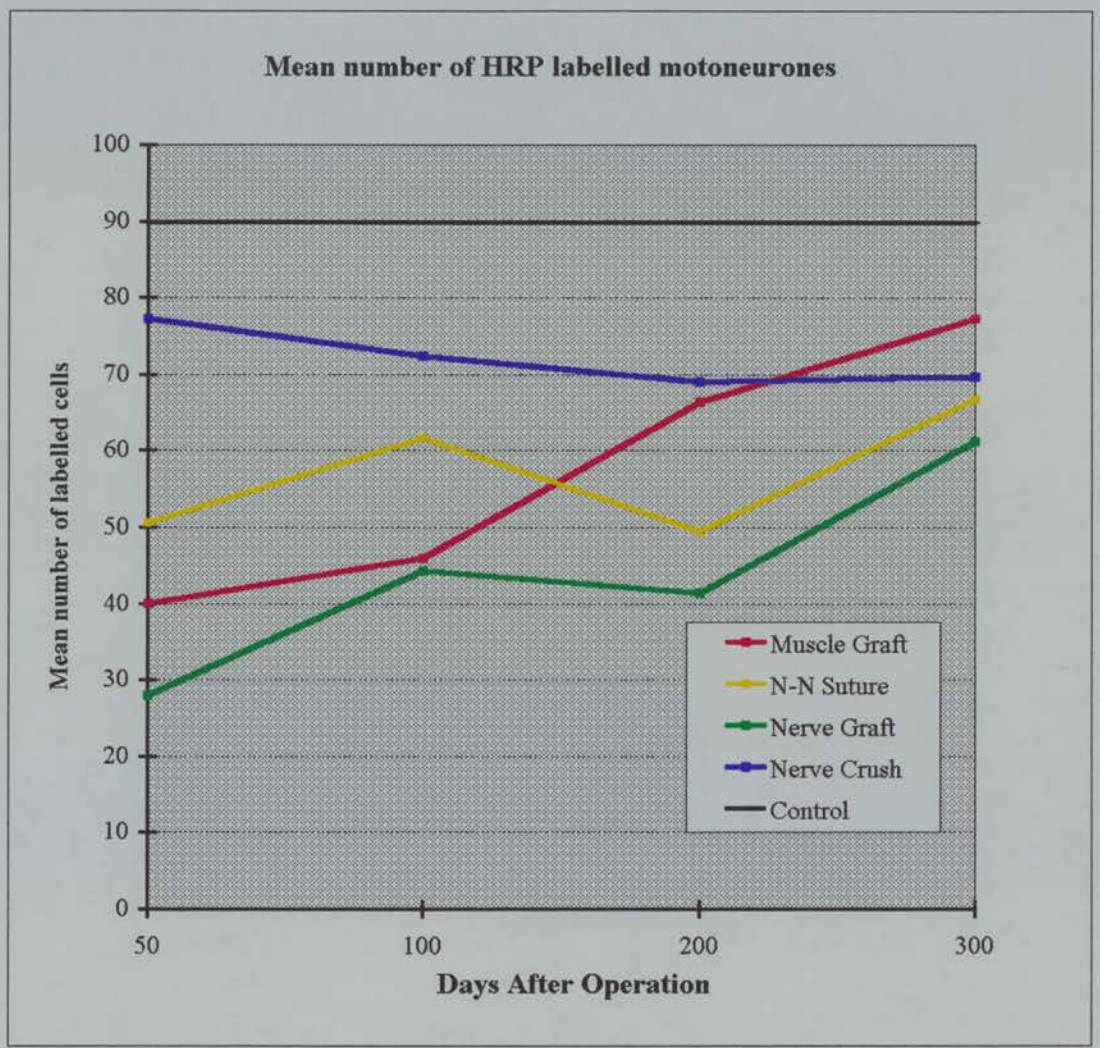

Figure 4.2.5 - Changes in the mean number of HRP labelled motoneurones at L3 to L5 level of spinal cord after each method of injury and repair and at each time period after operation.

After repair by means of a direct epineurial suture the number of labelled cells changed from 56.21% at 50 days to 68.53%, 54.98% & 74.26% after 100, 200 and 300 days respectively. By comparison the reduction caused by the nerve crush remains much less marked, ranging from 86.00% of control values at 50 days to 80.59%, 76.85% & 77.64% at 100, 200 & 300 days respectively. The increase in the number of labelled cells with time did not reach statistical significance after a nerve crush injury or after repair by direct epineurial suture.

4.3 Postoperative changes in the minimum diameter of spinal motoneurones.

Figure 4.3.1 and table 4.3.1 show the changes in the mean diameter of labelled motoneurones in the spinal cord after each method of injury and repair and at each time period after operation. Tables 4.3.2 and 4.3.3 show whether there is a significant difference in the mean diameter of labelled cells found after each of the methods of injury and repair and at each time period after operation, either when compared to one another or to control values. Significance was assessed at the 5% level. Initially, the mean diameter of labelled cells in the spinal cord was greater after each of the four methods of injury and repair than in the control animals. On comparison with the mean diameter of labelled cells in the control animals there was an increase in the diameter of 5.46% after a nerve crush injury, 9.39% after repair with a muscle graft, 8.06% after a nerve graft and 6.11% after a nerve to nerve suture. This increase in cell diameter reached statistical significance after the nerve crush injury ($p<0.05$) and repair with a nerve graft ($p<0.05$). The differences found between the mean cell diameter after each of the methods of injury and repair did not reach statistical significance when compared to one another. The

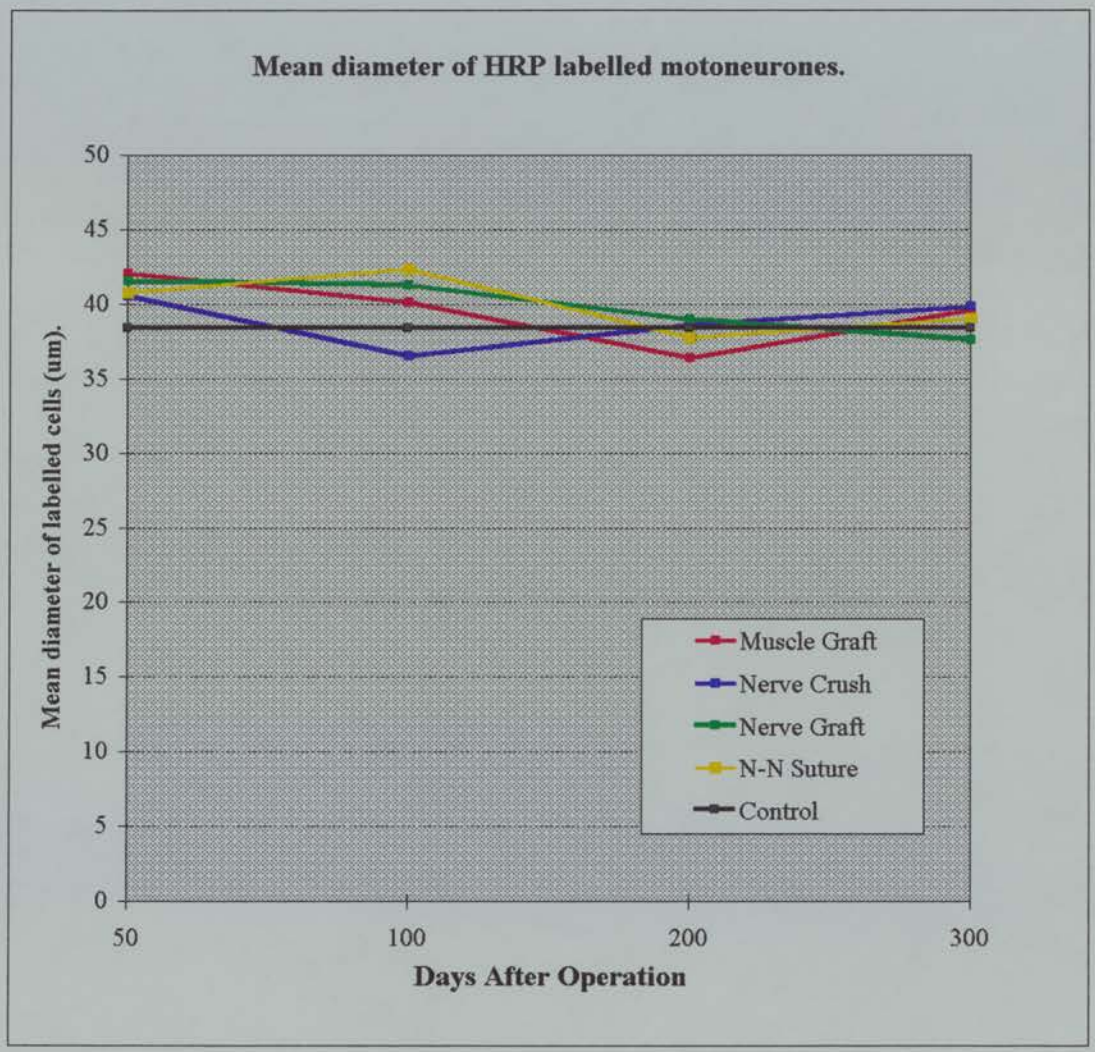

Figure 4.3.1 - Changes in mean cell diameter of HRP labelled motoneurones at L3 to L5 level of spinal cord after each method of injury and repair and at each time period after operation.

Repair	Mean diameter (μm)of labelled motoneurones			
	50 days	100 days	200 days	300 days
Muscle Graft	42.04 ± 4.11	40.14 ± 2.73	36.43 ± 2.54	39.60 ± 1.37
N-N Suture	40.53 ± 1.38	36.53 ± 0.78	38.63 ± 3.60	39.84 ± 3.05
Nerve Graft	41.53 ± 2.73	41.29 ± 3.62	38.97 ± 4.12	37.61 ± 0.87
Nerve Crush	40.78 ± 2.43	42.37 ± 3.02	37.80 ± 2.54	39.05 ± 1.32
Control	38.43 ± 1.17	38.43 ± 1.17	38.43 ± 1.17	38.43 ± 1.17

Table 4.3.1 - The mean diameter (± standard deviation) of HRP labelled motoneurones at L3 to L5 level of spinal cord after each method of injury and repair and at each time period after operation.

(A)

Repair	50 Days				
	Control	Muscle Graft	Nerve Crush	Nerve Graft	N-N Suture
Control	*	*	*	*	*
Muscle Graft	N.S.	*	*	*	*
Nerve Crush	P<0.05	N.S.	*	*	*
Nerve Graft	P<0.05	N.S.	N.S.	*	*
N-N Suture	N.S.	N.S.	N.S.	N.S.	*

(B)

Repair	100 Days				
	Control	Muscle Graft	Nerve Crush	Nerve Graft	N-N Suture
Control	*	*	*	*	*
Muscle Graft	N.S.	*	*	*	*
Nerve Crush	P<0.05	P<0.05	*	*	*
Nerve Graft	N.S.	N.S.	P<0.05	*	*
N-N Suture	P<0.01	N.S.	P<0.01	N.S.	*

(C)

Repair	200 Days				
	Control	Muscle Graft	Nerve Crush	Nerve Graft	N-N Suture
Control	*	*	*	*	*
Muscle Graft	N.S.	*	*	*	*
Nerve Crush	N.S.	N.S.	*	*	*
Nerve Graft	N.S.	N.S.	N.S.	*	*
N-N Suture	N.S.	N.S.	N.S.	N.S.	*

(D)

Repair	300 Days				
	Control	Muscle Graft	Nerve Crush	Nerve Graft	N-N Suture
Control	*	*	*	*	*
Muscle Graft	N.S.	*	*	*	*
Nerve Crush	N.S.	N.S.	*	*	*
Nerve Graft	N.S.	N.S.	N.S.	*	*
N-N Suture	N.S.	N.S.	N.S.	N.S.	*

Table 4.3.2 - Significance values for the comparison of the mean diameter of HRP labelled motoneurones at L3 to L5 level of spinal cord after each method of injury and repair at (A) 50 days, (B) 100 days, (C) 200 days and (D) 300 days after operation.

(A)

Duration	Muscle Graft			
	50 days	100 days	200 days	300 days
50 days	*	*	*	*
100 days	N.S.	*	*	*
200 days	P<0.05	P<0.05	*	*
300 days	N.S.	N.S.	N.S.	*

(B)

Duration	Nerve Crush			
	50 days	100 days	200 days	300 days
50 days	*	*	*	*
100 days	P<0.01	*	*	*
200 days	N.S.	N.S.	*	*
300 days	N.S.	P<0.05	N.S.	*

(C)

Duration	Nerve Graft			
	50 days	100 days	200 days	300 days
50 days	*	*	*	*
100 days	N.S.	*	*	*
200 days	N.S.	N.S.	*	*
300 days	P<0.05	N.S.	N.S.	*

(D)

Duration	N-N Suture			
	50 days	100 days	200 days	300 days
50 days	*	*	*	*
100 days	N.S.	*	*	*
200 days	N.S.	P<0.05	*	*
300 days	N.S.	P<0.05	N.S.	*

Table 4.3.3 - Significance values for the comparison of the mean diameter of HRP labelled motoneurones at L3 to L5 level of spinal cord at each time period after injury and repair with a (A) muscle graft, (B) nerve crush, (C) nerve graft and (D) nerve to nerve suture.

general trend was for a decrease in the mean cell diameter from 50 to 100 days after operation although the values mostly remained greater than those of the control. The nerve to nerve suture group was an exception as there was a slight increase in mean cell diameter (10.25%). This increase reached a statistical significant level when compared to the mean diameter of labelled cells in the control animals ($P < 0.01$). The decrease in mean cell diameter after a nerve crush injury resulted in a mean value which was less than that of the control group, a decrease of 4.94%. This difference reached statistical significance at the 5% level. Indeed, the mean cell diameter found 100 days after a nerve crush injury was significantly less than after any of the other methods of injury and repair ($p<0.05$ in case of muscle graft and nerve graft and $p<0.01$ in case of nerve to nerve suture). From 100 days to 200 days after operation there was a further decrease in the mean cell diameter of all groups with the exception of the nerve crush group. The increase in the mean cell diameter 200 days after the nerve crush injury brings the value closer to the range of mean cell diameters found after the other methods of injury and repair. The mean diameter of labelled motoneurones found after each of the methods of injury and repair was very similar to (within 5%) the values obtained for the control animals. The differences found between the mean cell diameter after each of the methods of injury and repair did not reach statistical significance either when compared to one another or with the control values ($p>0.05$ in each case). By 300 days after operation the mean diameter of the labelled motoneurones found after each of the methods of injury and repair was very similar and always within 4% of the control values. When compared with one

another and with control values these differences in mean cell diameter did not reach statistical significance ($p > 0.05$ in each case).

There was no consistent difference between each of the methods of injury and repair and the subsequent mean diameter of labelled motoneurones, at each of the time periods after operation.

4.4 Comparison of the results after the different methods of injury and repair.

Figures 4.2.1 - 4.2.4 indicate the different influences each of the methods of repair has on the motoneurone population and as would be expected, the nerve crush regularly resulted in the loss of fewer motoneurones than any of the three injuries involving transection of the nerve. Direct epineurial suture initially preserved a greater number of labelled cells than either of the grafting methods. The most significant result arises from the comparison of the nerve graft and muscle graft figures since these involve the same gap in the nerve to be bridged and same number of suture lines in the repair. There was some qualitative variation in the results with a consistent finding that more motoneurones became labelled where muscle grafts were used. However this finding was not statistically significant ($p > 0.05$) and should not therefore be taken to imply a different potential clinical outcome.

DISCUSSION

After the surgical repair of peripheral nerves, there is a slow but incomplete return towards normality in terms of function, anatomical features and physiological characteristics - the clinical relevance is discussed in chapter 10 (Sanders and Whitteridge 1946, Cragg and Thomas 1964, Mackinnon et al 1986, Glasby et al 1986a, Glasby et al 1986b, Glasby et al 1986c, Glasby et al 1988a). This is in keeping with the present study where all rats showed progressive signs of recovery up to 300 days after operation, by which time 92% of all animals exhibited the toe spreading reflex (Young 1942). Those animals who failed to regain their toe spreading reflex were found to have fixed ankle flexion which prevented any movement. Brushart (1990) obtained a similar result after the regeneration of the sciatic nerve across an 8mm gap, which was encased in mesothelial tubes. In each of the recorded cases of fixed ankle flexion the method of repair implemented has been one which tends to result in a prolonged period of regeneration (two suture lines in the case of the grafts and regeneration over a gap in the work of Brushart). Although this may appear to be a major disadvantage to the use of such methods of repair it must be remembered that it is unlikely in the human where routine physiotherapy following such a surgical procedure would tend to ensure its prevention.

5.1 Postoperative changes in number of spinal motoneurones associated with EDL

The chromatolytic changes which occur in the cell body after peripheral nerve injury have been well documented in the literature (see introduction). There is general agreement that such changes are most pronounced between one and three weeks after nerve section and that any peripheral neurones which are going to die and degenerate will do so in the first five weeks after injury (Lieberman 1971, Peyronnard and Charron 1986). The neurones whose axons have successfully regenerated, subsequently recover from chromatolysis. The factors which affect the degree of chromatolytic change and cell loss have been considered in greater detail in the introduction. It is generally accepted that mammalian spinal motoneurones return to normal after neurotmesis if their regenerated axons successfully reinnervate appropriate peripheral targets (Bowe et al 1992). However it has now been suggested that the perikaryon may undergo delayed changes, such as increased area, thicker dendrites and clumped distribution, up to one year after regeneration is complete (Bowe et al 1988, Bowe et al 1992). The extent of cell loss is much less marked in cases where the nerve has been repaired and allowed to regenerate (Weiss et al 1945, Lieberman 1971), however even in such cases muscle reinnervation is found to be abnormal not only in degree but in specificity (Brushart, Tarlov and Mesulam 1983, Brushart 1991).

The number of motoneurones claimed to innervate a particular muscle varies considerably among authors. The EDL in the rat is said to be innervated by anything from 81 (Nicolopoulos-Stournaras and Iles 1983) to 142 (Peyronnard et

al 1986) motoneurones. The current study found a mean of 89 (range 86 to 92) labelled motoneurones associated with EDL in normal control animals. The great variation in number is likely to be a reflection of the accuracy of the different methods of assessment. The method of administration of the HRP, the fixation concentrations and procedures, the enzymatic reaction, the storage protocol, the method of assessing labelled cells and the stereological methods applied all have consequences on the accuracy of the assessment (discussed in chapter 5.6). Consequently caution must be used in comparing the figures from different studies which have used different methods of assessment. To avoid such possible errors and to make the results of the current study directly comparable with the work of others, the cells labelled after injury and repair are expressed as a percentage of the normal population of cells.

The number of functioning motoneurones present in the spinal cord, after peripheral nerve repair, has been found to be significantly less than normal (Brushart et al 1980,1983). These findings concur with those in the current study. Indeed, the findings of Brushart et al (1980) that the motoneurone population was 69% of its normal value, three months after repair by direct epineurial suture, compares very well with the results of the current study (68.53% at 100 days after the same repair). This level of agreement between the results of the two studies is indicative of accurate HRP labelling since it is unlikely that inconsistencies in labelling between the two different techniques would lead to such similar results.

5.2 Comparison of the results after the different methods of injury and repair.

The failure of many anterior horn cells to regain peripheral connections must reflect inherent limitations in response to peripheral nerve injury, however this may be affected to some extent by the surgical technique (Brushart, Tarlov and Mesulam 1983). Brushart et al (1983) compared two different methods of repair, namely direct epineurial suture and individual fasicular suture. The use of individual fasicular suture produced significantly better results than the direct epineurial suture in terms of the accuracy of reinnervation however there was no significant difference in terms of numbers of labelled motoneurones between the two methods of repair. Again these findings are similar to those in the current study where there was no significant difference in the number of labelled motoneurones found after the different methods of repair which involved suturing, although this was not the case when compared with the nerve crush. Brushart et al (1983) explained this result by the fact that the epineurial suture allows the fasicles to change position within the epineurial sheath, which leads to subsequent misalignment. Similarly in the current study, the greater number of labelled motoneurones found in the spinal cord after a nerve crush injury could be attributed to the fact that neural alignment was maintained throughout. If neural alignment is maintained then this increases the likelihood that regenerating axons will re-enter the appropriate endoneurial tube in the distal stump. Consequently a greater number of motoneurones will re-form functional connections with their original target muscle. Neural alignment may also be a contributory factor to the

consistent, although not significant, differences found between the number of motoneurones present after each of the different methods of repair. The animals, in which the sciatic nerve was repaired by direct epineurial suture, consistently had a greater number of labelled motoneurones in the anterior horn of the spinal cord than either of the repair techniques which involved the use of a graft. This can be explained by the fact that the use of a graft results in the regenerating nerves having to negotiate two sets of suture lines, as opposed to the one involved in a direct epineurial suture. The mismatch of regenerating axons and appropriate endoneurial tubes occurs at the suture line after repair by direct epineurial suture. While some may re-enter appropriate endoneurial tubes many grow into unsuitable ones. This may result in the regenerating axons forming functional connections with muscles other than the original target muscle or, if a motor axon grows down a sensory pathway, not only will it fail to make functional connections but it will also block the way for the regeneration of appropriate axons. The insertion of a graft means that the regenerating axons must negotiate two sets of suture lines which are separated by a distance of 1cm. This leads to a greater degree of disorganization of the regenerating axons and thus the likelihood of re-entering an appropriate endoneurial tube is greatly reduced. Thus after repair by means of the insertion of a graft there are likely to be fewer functional connections and therefore fewer associated motoneurones in the spinal cord.

It was of considerable interest to find that repair of a nerve gap with a freeze thawed muscle graft was associated with the labelling of greater numbers of anterior horn cells than when a similar repair was carried out using a full thickness

nerve graft. This was in spite of the fact that the repairs were identical in every sense with the exception of the material used to form the graft. Although this difference in the number of labelled cells was consistent, it did not reach statistical significance. Despite the greater number of labelled motoneurones seen after repair by means of a muscle graft, previous studies (Myles and Glaby1991) have suggested that there is no difference between the muscle grafts and nerve grafts in respect of the recovery of function, electrophysiological and morphometric indices of recovery in the peripheral nerve. Thus although the muscle graft results in a greater number of motoneurones re-forming functional connections with the periphery this is not sufficient to cause any detectable difference in terms of the recovery of function

5.3 Alpha (α) and gamma (γ) motoneurones

α and γ motoneurones are intermingled within a single motoneurone pool (Bryan et al 1972, Burke et al 1977, Hongchien et al 1980, Carew and Ghez 1985, Peyronnard et al 1986). They are distinguished histologically by their difference in size and staining characteristics. Many authors have noted a bimodal frequency distribution of motoneurone size (Strick et al 1976 and 1977, Burke et al 1977, Nicolopoulas-Stournaras and Iles 1983, Peyronnard and Charron et al 1983, Peyronnard et al 1986). The γ motoneurones tend to have diameters of less than 35μm whilst the α motoneurones have a diameter anywhere between 35.0 and 80.0μm. The precise measurements defining the two groups vary between authors. Burke et al (1977) defined α motoneurones as measuring between 38.5μm and 78.0μm and γ motoneurones as measuring between 18.0 and 38.5μm however

Peyronnard et al (1986) were much less specific in defining γ as being less than 30.0 - 35.0μm and α as being greater. In studies using horseradish peroxidase as a neural tracer it has been noted by some authors that there is a difference in staining density between α and γ motoneurones. Strick et al (1976) and (1977) and Burke et al (1977) all observed that the most heavily HRP labelled cells were almost without exception the smaller motoneurones present, primarily from the γ motoneurone size range. Almost all cells in the γ size range were found to contain HRP granules of a larger diameter than those in the larger α motoneurones. This together with the greater density of granules is thought to result in the increased staining intensity. The level of staining intensity should never be used alone in the classification of α and γ motoneurones as other authors dispute these findings (Richmond et al 1978, Hongchien et al 1980, Peyronnard and Charron 1983). Strick et al (1976) and (1977) further subdivided the motoneurone pool according to size by distinguishing between large and small α motoneurones. Large α motoneurones were classified as being greater than 55.0μm in diameter and are thought to innervate fast twitch motor units whilst small α motoneurones have a diameter between 38.5 and 54.0μm and are thought to innervate the slow twitch motor units.

The successful operation of the motor system depends on the integrated output of the α and γ motor systems. The sensory feedback from the limb muscles plays an important part in their motor control (Barker et al 1993). The α motoneurones innervate the extrafusal muscle fibres whilst γ motoneurones innervate the intrafusal muscle fibres within the muscle spindles (Burke et al 1977,

Carew and Ghez 1985). The γ motoneurones innervate the muscle fibres at their polar regions where the contractile elements of the fibre are located (Carew and Ghez 1985). The two types of intrafusal muscle fibre are innervated by two different types of γ motoneurone. The γ dynamic motoneurone innervates the nuclear bag fibres and the γ static motoneurone innervates the nuclear chain fibres. The afferents which innervate the muscle spindles and the tendon organs monitor muscle stretch and tension. They provide the sensory feedback required for the reflex control of muscle contraction and its coordination (Barker et al 1993). The muscle contraction is due to the extrafusal muscle fibres which are innervated by the α motoneurones. Since the α and γ motoneurones are intermingled in the one motoneurone pool most descending systems which impinge on the α motoneurones also activate the smaller γ motoneurones ensuring that the two systems work together (Carew and Ghez 1985).

After the injury and repair of a peripheral nerve there is often a change in the relative number of different cell types present (Kuno et al 1974, Brushart and Mesulam 1980, Nicolopoulos and Iles 1983, Peyronnard et al 1986a,). It was noted by Carlson et al (1979) that after the amputation of the hindlimb in the cat the bimodal distribution of the cell diameter histogram altered. The position of the peak associated with larger diameter cells was displaced towards the peak associated with smaller diameter cells, by 4μm. The peak associated with larger diameter cells was also more dispersed than in the control. Peyronnard et al (1986) also noted a shift in the peak concentration of larger cells towards the smaller size range. The number of neurones with a diameter greater than 30μm was reduced

from an average control value of 72.1% to 46.7% after the permanent transection of the sural nerve. By contrast Brushart and Mesulam (1980) noted that after the transection and repair of the sciatic nerve the γ motoneurones only made up 2 - 3% of the total population whereas in control animals the γ motoneurones formed 16 - 22% of the population. Some have suggested that this decrease in the number of cells in the smaller size range was due to post-traumatic swelling, however since there is no compensatory increase in the larger diameter cell population this theory can be rejected. The depletion of the γ motoneurone pool was explained by Brushart and Mesulam (1980) as being the result of either the inability of γ motoneurones to regenerate over relatively long distances or the passing of insufficient time for the regeneration to occur. Brushart gave no consideration to the possibility that the γ motoneurones may not be adequately labelled with HRP and thus may be missed in any assessment made of the motoneurone pool (McHanwell and Biscoe 1981b). It is has been claimed by some that γ motoneurones do not take up HRP as readily as α motoneurones owing, at least in part, to their more isolated location on the intrafusal muscle fibres as opposed to the prominent location of the α motoneurones on the extrafusal muscle fibres. The consequences of the possible depletion of the γ motoneurone pool have not been fully established.

The minimum diameter of the α and γ motoneurones was measured, however as a consequence of problems associated with the image analysis system's hardware and software it was not subsequently possible to access these data. The only data available for analysis were the mean diameter of labelled cells found in

the spinal cord after each of the methods of injury and repair and at each time period after operation. Analysis of the mean diameter measurements hides details of the changes which occur in the relative proportion of the two cell types which are present, however since these were the only data available they were analysed to give an indication of the changes which occur in cell size. There was a general trend of an overall decrease in mean diameter of HRP labelled cells after each of the methods of injury and repair from 50 to 300 days after operation. After every method of injury and repair there was a significant decrease in cell diameter at some point between 50 and 300 days, for example the mean cell diameter after a muscle graft 200 days after operation was significantly less than at 50 days after operation ($p<0.05$). Likewise there was a significant decrease in mean cell diameter from 100 to 300 days after nerve crush ($p<0.05$) and nerve to nerve suture ($p<0.05$) and from 50 to 300 days after a nerve graft ($p<0.05$). At 50 days after operation the mean cell diameter was greater (up to 10%) after any method of injury and repair than in the control animals. This reached statistical significance on comparing control values with the results after a nerve crush or repair with a nerve graft ($p<0.05$). It is possible that the increase in mean cell diameter 50 days after operation was the result of post-traumatic swelling, which subsequently subsided resulting in the mean diameter measurements which were very similar to control values (within 4%) by 300 days after operation. This is unlikely, as discussed above, but cannot be disregarded without further information on the changes in both the α and γ motoneurone populations. Carlson et al (1979) and Peyronnard et al (1986) noted that in a cell diameter distribution histogram plotted after

transection of a nerve there was a shift in the position of the peak associated with larger diameter cells towards the peak associated with smaller diameter, i.e. there was a decrease in cell diameter of α motoneurones. Thus it would seem unlikely that the increase in mean cell diameter noted in the current study would be the result of an increase in the diameter of α motoneurones. The net increase noted in the current study is more likely to be a consequence of a decrease in the number of γ motoneurones labelled, as reported by Brushart and Mesulam (1980). By 200 and 300 days after each type of injury and repair the mean diameter of labelled cells lies within a very compact range around the control value, indeed these values are not statistically significantly different from the control. The fact that the mean diameter measurements were within 4% of, and not significantly different from, control values 300 days after operation suggested that the changes which occurred, in the size and relative number of each motoneurone type present, decreased with time. Thus if the increase in mean cell diameter 50 days after operation, was a consequence of a decrease in the number of labelled γ motoneurones, then it suggests that by 300 days after operation the γ motoneurone pool had regained its former complement of cells. If this is so then it would suggest that the depletion of the γ motoneurone pool described by many authors can be attributed to the passing of insufficient time for the regeneration to occur rather than to the inability of γ motoneurones to regenerate over relatively long distances or to their failure to take up and transport HRP.

There was no significant difference between the mean cell diameters found after each of the different methods of injury and repair with the exception of the

nerve crush group at 100 days. This is likely to be because of the unusually small mean diameter measurement associated with the nerve crush group. There was a variance of only 0.62 about the mean cell diameter of the 100 day nerve crush group thus the unusual result was accurate and not a consequence of an irregular result distorting the mean. This result is not consistent with the trends in mean diameter shown for all other repairs or for the nerve crush group at all other time periods. There was no consistent difference between the different methods of injury and repair and the changes in the mean diameter of the labelled cells, thus the changes are likely to be a consequence of the trauma of the injury rather than the method of repair.

5.4 Post-operative changes in the position of the spinal motoneurone pool associated with EDL.

The normal topography of specific groups of motoneurones in the spinal cord is well established (Sherrington 1892b, Romanes 1951, Swett et al 1970, Burke et al 1977, Brushart et al 1980 and 1983, Stournaras and Iles 1983, Peyronnard and Charron 1983 and 1986). The results of the current study, that the labelled motoneurone pool associated with EDL in the rat is located within the L3, L4 and L5 regions of the spinal cord, is in general agreement with the normal topographical position previously established for the motoneurones associated with this muscle. However, there appears to be a shift in the peak concentration of labelled cells between the control and experimental populations. In control animals the cells formed a compact population, concentrated primarily in lower L4 and L5,

whereas in experimental animals the cells regularly extended from upper L3, through L4 to L5. It is important to clarify that the aforementioned shift in topographical position of the motoneurone pool is apparent and not real. When the sciatic nerve is transected and repaired undirected growth of the axon sprouts leads many regenerating axons to muscles other than those they previously innervated. Thus a pool of motoneurones which previously served one muscle will subsequently control motor units scattered among several muscles (Mark 1969). Similarly, after reinnervation, the motoneurones which supply the target muscle will previously have served other muscles. Consequently, when the target muscle is injected with a neural tracer its labelled motoneurone pool will be found in areas of the spinal cord previously occupied by the motoneurone pool of another muscle. This change in the apparent distribution of spinal motoneurones supplying a particular muscle after reinnervation, is what is commonly known as the shift in topographical position. In accordance with the results of the current study, a similar shift in the topographical position of the motoneurone population was also found by Brushart and Mesulam (1980) following the repair of a peripheral nerve by means of a direct epineurial suture. Mallonga et al (1991) studied the effect of sciatic nerve transection on rats at 17 days gestation and found a greater shift in the motoneurone pool from L3-L5 to L2-L4. Myles et al (1992) found a corresponding shift in the topographical position of the cell bodies of the afferent fibres from EDL. In control animals the muscle afferent cell bodies were found predominantly in the L4 and L5 dorsal root ganglia - corresponding to the muscle efferent cell bodies which were found predominantly in the L4 and L5 regions of

the spinal cord. After nerve repair the afferent cell bodies were found throughout the L3 to L6 dorsal root ganglia - corresponding to the population of efferent cell bodies which were found to extend from upper L3 to L5. Thus there is a similar shift in the topographical position of the cell bodies of both the muscle afferents and muscle efferents.

The shift in topographical position of the labelled motoneurone pool associated with a particular muscle, after the injury and repair of its peripheral nerve, was assessed in more detail by Brushart et al (1983), Brown and Hardman (1987), Aldskogius et al (1987), Brushart (1990) and Brushart (1991). All authors noted whether the HRP labelled motoneurones were within the normal motoneurone pool location for that muscle. Aldskogius et al (1987) noted that 4 months after inflicting a lesion (freezing) on the rat sciatic nerve there was no shift in the topographical position of the motoneurone pool. Five weeks after crushing the sciatic nerve of the rat, Brown and Hardman (1987) found only 3% of labelled motoneurones outwith the normal topographic position. Similarly in the current study, after a nerve crush injury the topographical shift in the motoneurone pool was much less marked than after injuries involving the transection of the nerve. The results suggest that after injury by means of crush or freezing, almost all of the axons regenerate and reach the original peripheral innervation territory. This is made possible by the fact that neural alignement is maintained after such injuries. After the transection and repair of the rat sciatic nerve by means of a direct epineurial suture, 25% of the labelled motoneurones were outwith the normal position (Brushart et al 1983) as compared to 6% after individual fascicular suture

(Brushart et al 1983) and 53% after repair with mesothelial tubes (Brushart 1990). The transection of the nerve results in the loss of neural alignement and consequent repairs result in the mismatch of appropriate proximal and distal axon stumps. The results of Brushart et al (1983) showed that the number of labelled cells which were outwith their normal position after repair by means of a direct epineurial suture was significantly greater than after individual fascicular suture ($p < 0.001$). It has since been concluded that reinnervation using individual fascicular suture is significantly more accurate than other forms of repair (Brushart 1991).

It is unfortunate that in the current study the labelled motoneurones were not scored as in or out of the normal location for that motoneurone pool as in the afore-mentioned studies. The postoperative anatomical changes were only assessed qualitatively. If a quantitative assessment had been made of the degree of apparent topographical shift, it would have given a greater insight into the accuracy of reinnervation attained following the different methods of surgical repair. Since the accuracy of reinnervation is vital for the return of any useful degree of function it would be valuable to know whether one method of repair produced significantly more accurate reinnervation than another. This is especially true of the different methods of grafting since the previous studies failed to address this issue. It is well established that repair of a peripheral nerve involving the insertion of a graft results in poorer recovery of function than after the simpler repair by direct epineurial or individual fascicular suture. Repair involving the insertion of a graft involves two suture lines in the repair which increases the likelihood of mismatch of regenerating axons and appropriate endoneurial tubes. Therefore there is an

increased likelihood that the target muscle will be reinnervated by axons associated with inappropriate motoneurones. Such inaccurate rewiring, after reinnervation, results in the motoneurone pool which previously served one muscle subsequently controlling motor units which are scattered among several muscles. Thus attempts to contract one muscle may result in the weak contraction of a whole group. If the original motoneurones reinnervate muscles which are antagonistic to the original target muscle obviously attempts to contract the target muscle will result in abnormal movements. In the case of repair by means of a direct epineurial suture or an individual fascicular suture such mismatch still occurs but, theoretically, to a lesser degree since there is only one suture line in the repair. It would be interesting to assess if one method of grafting resulted in a greater degree of accuracy in reinnervation as this would obviously influence the subsequent functional recovery. The failure to assess the labelled motoneurones in this way was a shortcoming of this study and is one aspect of the work which ought to be followed up.

5.5 Conclusion

The results of the current study indicate that whilst a small portion of the motoneurone population does die following peripheral nerve surgery, this is not a significant number. The majority of the anterior horn cells appear to have the ability both to survive nerve transection and to form new functional connections with the muscles after repair. However as a consequence of the mismatch of regenerating axons and appropriate endoneurial tubes, the spinal motoneurones frequently reinnervate muscles other than the original target muscle. This can have great consequences on the recovery of function. The degree of cell loss is

influenced to a certain extent by the nature of the injury and the method of repair. Injuries involving neurotmesis result in the loss of a greater proportion of the cell population than the less severe injuries involving axonotmesis. In cases where the severed nerve has been repaired by means of a direct epineurial suture a greater proportion of the motoneurone population is preserved than after repair by means of a graft. The two methods of grafting produced comparable results, although the muscle graft tended to result in the preservation of a greater number of cells - this was not statistically significant at the 5% level. It would appear that the muscle graft is a feasible alternative method for the surgical repair of peripheral nerves, capable of producing comparable recovery whilst avoiding many of the disadvantages.

The changes in morphology seen at the spinal level on reinnervation after each of the methods of injury and repair do not correlate with the results of previous studies (Glasby et al 1986a, Glasby et al 1986b, Glasby et al 1986c, Glasby et al 1986d, Glasby et al 1988a, Glasby 1990, Grieve et al 1991) which assessed the recovery of function after the same repair methods. Therefore it seems that the failings of any given method of nerve repair are more likely to be defined by the events at the periphery than those occurring centrally, where a fairly uniform response is to be expected. The clinical relevance of these findings will be discussed in chapter 10

5.6 The problems associated with the use of the neural tracer horseradish peroxidase.

Horseradish peroxidase neurohistochemistry has become one of the most frequently used methods for tracing neural connections within the central nervous system (Mesulam 1978). However, despite its great popularity, there is still a great deal of debate about its accuracy and reliability. A great many studies have been carried out on the degree of accuracy of HRP labelling, with the results claiming it to label anything from only 70% (Stournaras and Iles 1983) to 98.3% (Peyronnard and Charron 1983) of the motoneurone population. One reason put forward for this discrepancy is that after nerve section injured cells do not take up or transport protein tracers (like HRP) as well as normal nerves (Kristensson and Olson 1973, Kristensson and Olson 1975, Peyronnard et al 1986b). This is thought to be the result of changes in the transport mechanisms within the neurone following axotomy and continues for as long as regeneration is prevented (Peyronnard et al 1988). However this failure of uptake and transportation of HRP is not thought to be of sufficient degree, in longer term experiments where the injured nerve has been allowed to regenerate, to lead to inaccurate cell counts (Myles et al 1992).

Despite the reservations about the accuracy of HRP labelling it is now being used to assess the accuracy of new neuronal tracers such as dextran amine coupled to rhodamine (Fritzsch and Sonntag 1991). The new tracer was deemed to be a success since the number of motoneurones labelled was not significantly different from numbers obtained after labelling with HRP.

One of the most debated aspects of HRP methodology has been the most

appropriate method of administration, with authors trying a variety of techniques such as intramuscular injection (Burke et al 1977, Richmond et al 1978, McHanwell and Biscoe 1981, Janjua and Leong 1981, Mesulam 1982), application of solid crystals to the cut end of nerve (Gottschall et al 1980, Neuheuber and Nierderle 1980, Stillhard 1981, Stournaras and Iles 1983), insertion of cut nerve into capillary of aqueous solution (Winer 1977, Richmond et al 1978, Baulac and Meininger 1979, Stournaras and Iles 1983), injection of aqueous solution into the proximal stump (Fernandez et al 1990), application of crystals onto crushed area of a nerve (Farel et al 1989) implantation of solid pellets of HRP (Griffin et al 1979) or the implantation of slow release HRP gels (Griffin et al 1979). In the current study, an intramuscular injection of HRP was favoured. The main objections previously given against its use were concerned with false labelling which occurred as a result of its tendency to diffuse to surrounding tissues. The work of Janjua and Leong (1981) is widely quoted in the literature to substantiate this claim, however most authors have failed to recognize that whilst that is indeed true in the neonatal and developing animal it was shown not to be so in the adult. In the current study the use of intramuscular injection of the enzyme was chosen as the preferred method of administration since it was found to be quick and easy, it minimized the amount of dissection required, minimized the duration of anaesthesia and produced no false labelling of surrounding motoneurone populations (as indicated by controls). Similarly many of the recent studies using HRP have used intramuscular injection as the administration method of choice (Mallonga et al 1991, Scarisbrick et al 1992, Angelov et al 1993, Tsukazaki et al 1993, Borke 1993).

5.6.1 Sensitivity and stability of HRP labelling.

Horseradish peroxidase is a popular neuroanatomical tracer, partly because simple sensitive histochemical procedures can accurately localize its presence (Weinberg and Van Eyck 1991). These procedures strive to minimize artefact, optimize specificity, sensitivity and tissue preservation (Weinberg and Van Eyck 1991) whilst ensuring safety, reliability and cost effectiveness. In the last 15 years, literature on HRP histochemical procedures has been produced in abundance. Unfortunately, the majority of these reports presented conflicting opinions as to the most appropriate technique, resulting in a great deal of confusion. Hence it was felt that an unbiased synopsis of the literature was required.

(a) Sensitivity and the choice of chromagen.

The success of HRP as a neural tracer depends, to a certain extent, on the sensitivity of the reaction which allows its visualization. There is a large number of chromagens which have been used in the HRP reaction in recent years, with variable degrees of success. The most popular chromagens currently in use include tetramethyl benzidine (TMB), diaminobenzidine (DAB), Hanker Yates, o-dianisidine (O-D), o-tolidine (O-T), benzidine dihydrochloride (BDHC), p-phenylenediamine and pyrocatechol (PPD-PC), ammonium molybdate and ammonium paratungstate (Mesulam and Rosene 1979, Morrell et al 1981, Olucha et al 1985, Weinberg and Van Eyck 1991). Each of these chemicals has relative advantages and disadvantages in terms of its success as a chromagen. A successful chromagen ought to be sensitive, stable, safe (non carcinogenic) and reliable. Since the chromagen chosen for HRP visualization is critical to the completeness of the

neuroanatomical findings (Morrell et al 1981) it is important to make the right choice. There is a general trend in the literature that studies carried out in the 1970's used either DAB or BDHC as the chromagen (Halperin et al 1975, Strick et al 1976, Mesulam 1976, Adams 1977, Burke et al 1977, Hanker et al 1977, Rosene et al 1978, Molin et al 1978). In 1978 Mesulam introduced the TMB visualization method which further confused the issue. Consequently a series of comparative studies was set up in order to assess which chromagen was the most sensitive in terms of HRP visualization (Mesulam 1978, Mesulam and Rosene 1979, Morrel et al 1981). These studies are in agreement that the TMB procedure produces distinctly superior results than the other chromagens. As a consequence of these results, in most subsequent studies TMB was the chromagen of choice (Mesulam et al 1979, Brushart and Mesulam 1980, Brushart et al 1983, Peyronnard et al 1983, Peyronnard et al 1983, Nicolopoulos et al 1983, Schmalbruch 1984, Peyronnard et al 1986, Peyronnard et al 1986, Wada et al 1990, Brushart 1991, O'Hanlon and Lowrie 1991,).

(b) Safety

TMB was introduced for its probable lack of carcinogenicity (Weinberg and Van Eyk 1991) since most of the alternative chromagens, including DAB, BDHC, O-T, O-D and dichlorobenzidine, are carcinogenic (Mesulam 1978). Although TMB is not thought to be carcinogenic it has a number of other harmful effects, some of which are thought to be irreversible. It is harmful by inhalation, ingestion, in contact with the skin or eyes and is regarded as a possible mutagen. Appropriate precautions must be taken when handling the chemical.

(c) Sensitivity and experimental protocol.

The superiority of the TMB technique, as a method of visualizing HRP labelling, is largely due to the choice of chromagen. However it is also influenced by the method of fixation, the storage after fixation, incubation specifications, the post reaction handling and cleanliness of equipment (Mesulam and Rosene 1979).

1. Fixation

The principle function of fixation is to stabilize and preserve tissue so that its normal structure is not destroyed by endogenous autolytic enzymes or other catabolic activities (Hopwood 1969, Van Diujn 1973). For enzyme histochemistry, fixation must also limit the diffusion of enzymes from their *in vivo* location by binding them to the tissue (Rosene and Mesulam 1978). It was reported by Adams (1977) that fixation should be with glutaraldehyde alone since glutaraldehyde stabilizes HRP but formaldehyde reduces HRP activity. This is in agreement with the findings of Jones et al (1974) and Kim et al (1976), however Janigan (1964), Anderson (1967), Hopwood (1967) and Janigan (1975) found the converse to be true. Rosene and Mesulam (1978) investigated the problem of fixatives and concluded that both aldehydes depress HRP activity. They found that this was minimized if 1.25% glutaraldehyde and 1% paraformaldehyde were used. Since the process of fixation depresses the activity of most enzymes a compromise must be reached between tissue stabilization and enzyme inactivation (Rosene and Mesulam 1978). Both the degree of fixation and the degree of enzyme suppression are dependent on the concentration of the fixative, the temperature of the fixative and the duration of its contact with the tissue (Rosene and Mesulam 1978). The

fixative used in the perfusion should be used at 21° C as this facilitates the diffusion of the fixative from the capillary system into the tissue. Flushing with sucrose buffer after fixation should be conducted at 4° C in order to cool the tissue as rapidly as possible and reduce the possibility that unsupressed autolytic enzyme activity would disrupt either the tissue or the HRP (Rosene and Mesulam 1978). This flush with sucrose buffer aids in the removal of excess fixative from the system thus terminating the fixation process. This procedure limits the exposure of the HRP molecules to unbound aldehydes and hence reduces the risks of denaturation. Over exposure to fixatives, for as little as one hour, significantly decreases the number of labelled cells subsequently seen (Rosene and Mesulam 1978). The exposure time of the tissue to fixative during the perfusion should not exceed 30 minutes.

2. Storage after fixation.

After fixation the tissue should be stored in 10% sucrose buffer at 4 - 10°C. Tissues can be stored in this was for up to 7 days with no apparent depression of sensitivity (Mesulam et al 1980). Since the sucrose buffer offers the tissues a degree of cryoprotection it is advantageous to store the tissues in this way prior to sectioning. In the current study the sections were never left for more than 20 hours.

3. Incubation specifications.

The sensitivity of the TMB procedure is significantly affected by the pH of the incubation medium. If the pH of the solution falls outwith the range 2.5 - 4 the sensitivity is reduced and HRP labelled cells are impossible to visualize (Mesulam

1978). The temperature of the incubation medium influences sensitivity, artefact and the size of TMB reaction product granules. An increase in temperature results in slightly enhanced sensitivity but also an increase in artefact and the size of reaction-product granules which obscure morphological details and specificity. If the temperature is decreased then the converse is true - better morphological detail but a significant decrease in sensitivity (Mesulam 1978). The time allowed for incubation is a compromise between the minimum time required for adequate sensitivity and prolonged exposure which increases the amount of artefact present (Mesulam 1978). In the current study if the timings recommended by Mesulam were strictly adhered to then the level of artefact was intolerable. When the solution was on the brink of changing from green to blue a large number of blue granules began to appear in the solution. If the sections were removed at this stage the staining was adequate, however if the sections remained in the solution for the full 20 minutes recommended by Mesulam the blue granules were deposited on the tissue.

The concentration of the chromagen and the hydrogen peroxide ought to be considered together to optimize sensitivity while preserving morphological specificity (Mesulam 1978). If the quantity or concentration of hydrogen peroxide is too high then artefact is liable to become a problem. In the current study a series of trials was set up in order to assess the volume of hydrogen peroxide required to produce adequate staining but minimum artefact. Although recommendations in the literature give a rough guide to the volumes required it is better to assess each experimental set up individually since a variety of external factors also have an

influence on the reaction.

The exposure of TMB to alcohol depresses sensitivity and increases the size of the reaction product granule however, since TMB is readily soluble in alcohol but not in water, its use is unavoidable (Mesulam 1978). The use of alcohol should be kept to an absolute minimum.

The enzymatic reaction is best carried out on free floating sections since there is severe depression of sensitivity once the sections have been slide-mounted (Mesulam et al 1980). Many of the studies in the literature fail to mention whether the sections used were free floating or slide-mounted. The degree of labelled cell loss resulting from the use of slide mounted sections is so great as to make the results meaningless.

4. Treatment after enzymatic reaction.

After the enzymatic reaction the sections can be left for up to 4 hours, before mounting, without a significant loss of sensitivity (Mesulam et al 1980). If left for more prolonged periods there is marked and rapid depression in the ability to demonstrate the presence of reaction product (Mesulam et al 1980). Mesulam et al 1980 stated that once slide mounted, the sections could be left at room temperature for at least seven days without any loss of reaction product. In the current study storage at this temperature resulted in a significant loss of labelled cells hence all sections were stored at 4°C in the dark as enzyme denaturation is greatly reduced in this way. Even storing the sections in this manner resulted in the loss of reaction product within a matter of weeks unlike the 10 months claimed by Mesulam (1978).

The exposure to alcohol is unavoidable during the dehydration process prior to applying a coverslip. A compromise had to be found between adequate dehydration of the tissue and the loss of reaction product (Mesulam et al 1980). A brief exposure of 10 seconds to a series of graded alcohols satisfies both requirements (Mesulam et al 1980).

5. Equipment

The TMB procedure is a very sensitive reaction. It is very important that all glassware, racks and incubation trays are impeccably clean as the slightest trace of contaminant can cause the reaction to fail. After washing all equipment associated with the reaction must be thoroughly rinsed in alcohol to remove any traces of detergent as this is also detrimental to the reaction. The glassware must be allowed to air-dry prior to use to ensure that all the alcohol has evaporated since excess alcohol causes the depression of TMB sensitivity.

(d) Stability

Mesulam (1978) stated that the majority of material maintained its dark blue colour at least for as long as 10 months, however this was not the case in the current study. Despite the care taken to ensure the sections were stored in the dark and at low temperatures there was a considerable loss of reaction product within a matter of weeks. All sections were assessed and the labelled cells measured and counted within 5 days of the reaction to minimize the likelihood of underestimation resulting from a loss of reaction product with time.

5.7 Accuracy of stereological techniques

In the current study a correction factor was applied to the crude cell counts, in order to minimize any errors introduced as a result of the inability to distinguish between whole cells and cell fragments. The technique, introduced by Abercrombie in 1946, has been extensively used by a great many scientists and is still regularly quoted in the literature today (Brushart 1990, Harsh et al 1991, Henery and Kaufman 1992). However in recent years the accuracy of his technique has become questionable, with many authors claiming that it makes unreasonable assumptions regarding the size and shape of the structures under sample (Mayhew 1979, Huag 1986, Gunderson 1986, Mayhew 1989, Mayhew 1993). Current thought favours the use of stereological techniques which are unbiased and independent of cell size, section thickness, particle distribution and plane orientation. The dissector and the fractionator methods of Gunderson (1986) are currently the two methods of choice.

Despite the current trend to favour alternative methods, Abercrombie's technique was still the method of choice for the current study. One of the main reasons for this decision was that when the project was initiated, the dissector and fractionator methods were very much in their infancy. Indeed, in 1988, Mayhew referred to Gunderson's technique as the new fractionator method. It was decided that it was a safer option to stick to a tested method rather than opting for something relatively new and not, at that stage, fully accepted. There was no way of foreseeing that the dissector and fractionator would indeed mark the new way forward in stereology.

Despite the great many advantages of the new techniques, they still have many limitations, some of which were not problems when using Abercrombie's method. In order to apply the fractionator method accurately it is necessary to have prior knowledge of the approximate number of cells one expects to find within the total tissue mass. Without this knowledge there is no way of calculating the number and size of windows required for sampling. These factors influence the efficiency and accuracy of the technique (Mayhew 1990). This requirement of prior knowledge could be very limiting and indeed in some cases could lead to the need to carry out a total count before the sampling technique could be applied accurately! There is a similar drawback with the use of the dissector since accurate knowledge of the absolute minimum size of particles present in the population is required. This information is required in order to calculate the required maximum distance between sections to ensure that the probability of large and small cells being present in a section is the same. Without this knowledge the technique can become inaccurate and biased (Pakkenberg and Gunderson 1988). Abercrombie's method requires no such prior knowledge about cell size or population size and hence it is quicker and simpler to apply

Many authors claim that it is far more efficient, both in terms of time and accuracy, to sample populations rather than to do total counts (Mayhew 1983, Mayhew 1988). This is undoubtedly true when the populations to be sampled are very large, however with smaller populations this is not necessarily the case. It has been stated that in order to apply any stereological technique accurately a minimum of 200 objects should be sampled. In the current study the cell population under

study was consistently less than 100, hence a total count was preferable to any sampling technique

The fractionator method involves exhaustive sectioning of the tissue, however it is important that the tissue is trimmed to ensure the removal of any areas which do not contain the cell population under test. In the current study this would have been a very difficult constraint to apply. Whilst it would be a relatively simple task to trim the normal side and the white matter, it would be impossible to trim the grey matter which surrounded the cell population, without serially sectioning the cord in order to assess the labelled cells' location. There is an additional complication in that although the population of motoneurones associated with the EDL appears as a discrete population in normal animals the location and distribution alters following injury and repair of the peripheral nerve. Hence in the current study it would not be possible to apply the fractionator method accurately

The main criticism of Abercrombie's technique is based on the fact that he made assumptions about the size and shape of the particles to be measured. It is claimed that the mean particle diameter measured was taken to be the true mean diameter, an obvious error since some cells would be sectioned through the equator and some through the poles (Mayhew 1979). This is an unfair criticism of Abercrombie since he built a correction factor for this error into his method. This took the form of a secondary formula which had to be calculated from sections taken at right angles to the originals, hence many authors failed to apply that part of the formula. The other main criticism is that he assumed that all particles to be measured were approximately spherical or ellipsoidal. This is also an unfair

criticism since he clearly emphasized the dangers of applying the technique to objects of different geometrical form.

Whether or not these errors are true is not really relevant in the current study since they are systematic errors which are consistent between groups and therefore can be ignored in a comparative study.

5.8 Future Research.

It would be very useful to carry out a quantitative assessment of the degree of topographical shift of the spinal motoneurone pool associated with a particular muscle after the injury and repair of its peripheral nerve. Although this has been assessed to a limited degree there has been no long-term comparative study which assesses the different effects of direct epineurial suture, nerve grafts and muscle grafts. It would be useful to assess the change in the topographical position of the motoneurone pool associated with a particular muscle after reinnervation using the technique of Harsh et al (1991). They used a double labelling technique which involved labelling the original motoneurone pool with DiI (1,1-dioctadecyl-3,3,3,3-tetramethylindocarbocyanine perchlorate), transecting and repairing the nerve and at a later time labelling the neurones which have regenerated and reinnervated the target muscle with a second marker - fluorogold. This technique would allow a precise assessment of the accuracy of reinnervation after each of the methods of injury and repair. This technique has one major disadvantage - the expense of the chemicals involved, thus although it may be a less accurate technique it may be a more feasible option to use the technique of Brushart et al (1983). Their technique involved labelling the motoneurones associated with a particular muscle with HRP

and scoring the labelled cells as in or out of the location normally occupied by that motoneurone pool in a control animal. The use of either technique would give an insight into the accuracy of reinnervation attained following the different methods of surgical repair. The importance of this issue is considered more fully in the discussion.

The current study dealt with immediate repair of the peripheral nerve only. It would be of considerable interest to compare the results obtained here with a similar study which involved a delayed repair. It is well established that immediate repair produces superior results compared to delayed repair however the latter is still widely used, either out of choice or necessity. It would be beneficial to know whether one method of repair produced better results than another in cases of delayed rather than immediate repair. If the shortcomings of delayed repair could be minimized or compensated for with the method of repair implemented then this could have a major effect on the degree of recovery achieved

Any technique which enhances the regeneration of an injured peripheral nerve is worth further consideration. In recent years transcutaneous low power irradiation and the delivery of an electrical field following a crush injury to the nerve have been found to have an effect on different aspects of nerve regeneration (Kerns et al 1992, Anders et al 1993). Treatment with lasers was found to increase the rate of regeneration of the rat facial nerve with a significantly greater number of HRP labelled motoneurones 9 days after irradiation of the crush injury than in untreated animals. The delivery of an electrical field after a similar injury did not have an effect on the number of motoneurones present but the nerve was said to

appear healthier and more normal looking and the enlarged endoneurial space was significantly reduced in treated animals (Kerns et al 1992). The effects of these techniques were only assessed following the relatively minor injury of a nerve crush, it would be interesting to assess whether or not they would enhance the recovery of transected nerves.

ALTERATIONS IN MUSCLE ARCHITECTURE AFTER PERIPHERAL NERVE INJURY AND REPAIR:

A QUANTITATIVE AND QUALITATIVE STUDY USING HISTOLOGICAL AND HISTOCHEMICAL STAINS

INTRODUCTION

6.1 The Structure of Muscle

Mammalian skeletal muscles are made up of closely packed cylindrical multinucleated muscle fibres which are bound together in a connective tissue framework (Williams and Warwick 1983). The connective tissue framework is made up of three layers - the epimysium which encloses the muscle and is continuous with the tendons which attach the muscles to the skeleton, the perimysium which subdivides the muscle fibres into bundles or fascicles and the endomysium which extends between the individual muscle fibres. The blood vessels and nerves which supply the muscle ramify these connective tissue layers. The individual muscle fibres are polygonal in shape when seen in transverse sections, as a result of being closely packed within the perimysium. The fibres many nuclei are situated just under its cell membrane, the sarcolemma, and tend to be elongated or ovoid in shape. The muscle fibres contain longitudinally orientated component fibrils called myofibrils which occupy 85 - 90 % of its volume. The individual myofibrils are striated and this gives rise to the pattern of striations in a skeletal muscle fibre. The skeletal muscles associated with the trunk and limbs are innervated by motor axons which are derived from motoneurones in the anterior horn of the spinal cord. A single motoneurone together with the motor axon derived from it and the many muscle fibres uniquely innervated by it is known as the motor unit. The motor unit is the elementary unit of neuromuscular function (Slater and Harris 1988)

6.2 Muscle Fibre Types.

Mammalian motor units contract at different speeds. In most muscles two distinct types of unit can be distinguished, 'slow twitch' or type I and 'fast twitch' or type II. This subdivision of muscles into the two fibre types was suggested by Dubowitz and Pearse (1960 a and 1960b). Type I fibres were characterized as having high oxidative and low glycolytic activity while the converse was true for type II fibres. Engel (1962) also devised a two fibre-type system. This system based the differentiation on myofibrillar ATPase activity, with type I fibres having low ATPase activity and type II high activity. Subsequent work by Sreter (1966), Samaha (1967) and Guth and Samaha (1969) showed that the stability of the ATPase enzyme was variable depending on the muscle fibre type and pH. Myosin of a slow muscle fibre was relatively more alkali labile and acid stable than that of a fast muscle fibre, whereas the converse was true for the myosin of a fast muscle fibre. This led to a further subdivision of type II fibres into type IIa and IIb. Type IIa fibres react strongly with ATPase at pH 10.2 but weakly at pH 4.6 and 4.3 whereas type IIb fibres react strongly at pH 10.2 and 4.6 but weakly at pH 4.3. (Brooke and Kaiser 1970)

Muscles which predominantly consist of one fibre type are classed as either fast or slow muscles and differ in their patterns of energy metabolism. Slow twitch muscle fibres rely on oxidative mechanisms for the generation of energy whereas fast twitch muscle fibres rely on glycolysis, either on its own or in combination with oxidative mechanisms (Slater and Harris 1988). Consequently, slow twitch fibres have more mitochondria and are more vascularized than fast twitch muscle

fibres (Eisenberg and Kuda 1976). The muscles ability to sustain contraction is dependent on the pattern of energy metabolism. Muscle fibres with oxidative metabolism are said to be 'fatigue resistant' as they are able to maintain substantial tension for some minutes, while those with glycolytic metabolism are said to be 'fatiguable' as they fatigue rapidly in the face of sustained stimulation (Slater and Harris 1988). Thus even though fast fibres are able to contract more rapidly, they also fatigue more rapidly and hence are better adapted for intermittent bursts of contractile activity.

The muscle fibre properties are determined largely by the motoneurones that innervate them (Gruber and Zenker 1978). Fast twitch muscle fibres are innervated by fast motoneurones and *vice versa* for slow twitch muscles. Fast motoneurones tend to be greater than 50um in diameter and stain strongly for Acetylcholinesterase (Ache) whereas slow motoneurones are approximately 45um in diameter and stain moderately for Ache (Odutola 1972). Slow motoneurones, as a group, show lower conduction velocities of their axons, higher input resistances and higher densities of homonymous and heteronymous group Ia synaptic terminals (Burke et al 1976). The long after-hyperpolarization in these slow motoneurones helps to ensure that the frequency of firing is relatively low. Conversely, fast contracting motor units are innervated by high threshold motoneurones with rapidly conducting action potentials which can occur in high frequency bursts. These fast motor units normally generate a greater tension than those innervated by slow motoneurones (Slater and Harris 1988).

The relative proportion of fast and slow motor units present varies greatly

between different muscles and is a reflection of the different functional demands made of the muscle. In the rat, the EDL is a fast twitch muscle which is made up of 90 to 97% type II fibres (Ariano et al 1973, Pullen 1977, Nierdele and Meyer 1978). The primary function of EDL is in the movement of the digits - a movement which generally requires rapid contraction but need only be sustained for short periods. By contrast the primary function of the slow soleus muscle is associated with the maintenance of posture, a role which requires tension to be maintained for prolonged periods. Consequently the soleus in the rat is made up 75 to 80% type I fibres (Ariano et al 1973, Pullen 1977, Narasuwa 1985). The relative proportion of each fibre type present is also dependent on a variety of other factors such as the age and species of the animal and whether or not the nerve which supplies it has been injured. The relative proportion of the two fibre types found in the soleus muscle of other species is markedly different from the figures quoted above for the rat, with type I fibres comprising 67% of the soleus in the mouse (McLachlan 1983), 89% in the human (Johnson et al 1973), 95 to 100% in the cat (Close 1972) and 100% in the guinea pig (Karpati and Engel 1968). This difference between species is likely to reflect the different demands made of the soleus muscle according to the different postural and locomotive activities of the different species. The progressive denervation and reinnervation of muscle fibres which accompanies the ageing process, eventually reaches a phase where the fibres become permanently denervated and are replaced by fat and fibrous tissue (Lexell et al 1986). This process involves the preferential denervation of type II fibres, hence there is a consequent change in fibre type distribution (Lexell et al 1986).

Similarly, after the transection and repair of a peripheral nerve, there is a change in the distribution and relative abundance of each fibre type present in the target muscle. These changes will be considered along with the other morphological alterations seen in the target muscle, after injury and repair of the peripheral nerve .

6.3 The response of muscle to neuronal damage.

1. Denervation.

When the nerve supplying a muscle is cut, nerve-stimulated contractility of the muscle is lost and atrophy ensues (Jaweed et al 1975). Within three weeks of denervation the neuromuscular junction is destroyed (Jaweed et al 1975). The muscle fibres tend to atrophy rapidly at first, the rate then slows down and eventually stabilizes with fibres at a much smaller size than before. This was studied in detail in the Australian opossum by Sunderland and Ray (1950), who found that the mean cross- sectional area of the denervated fibres decreased by 70% in the first 60 days; in the following 60 days it lost a further 10% and thereafter the cross sectional area remained at 10-20 % of its initial size. There is no constant relationship between the degree of atrophy and the duration of denervation (Jennekens 1982). The rate at which atrophy occurs varies not only between species but also between different muscles within the same animal and different fibre types within the same muscle (Bowden and Gutmann 1944, Jaweed et al 1975, Sunderland 1978, Jennekens 1982). Many authors agree that type II fibres atrophy earlier and more rapidly than type I (Bajusz 1964, Karpati and Engel 1968a, Riley and Allin 1973, Niederle and Mayr 1978) however other literature disagrees with this finding (Jaweed et al 1975, Pellegrino and Franzini 1963).

Dubowitz (1985) stated that selective atrophy is a common abnormality of almost all muscle diseases and that denervating diseases tend to result in twin peaked fibre diameter frequency histogram, i.e. 2 distinct populations of fibres, one larger in diameter than the other.

Denervated fibres appear to lose their polygonal shape and as atrophy proceeds, they become more round or oval in shape. There is an increase in the amount of connective tissue and fat found between the muscle fibres (Gutmann and Young 1944, Sunderland and Ray 1950, Grieve et al 1991), however there is a variation in the amount present in different individuals after a similar period of denervation (Bowden and Gutmann 1944). There is also a great deal of variation in the rate of connective tissue proliferation between different species (Willard and Grau 1924, Tower 1935, Bowden and Gutmann 1944, Gutmann and Young 1944). The amount of connective tissue and fat present increases with the duration of denervation, indeed if reinnervation of the muscle does not occur then these tissue components eventually replace the muscle fibres (Bowden and Gutmann 1944, Seddon 1975, Sunderland 1978).

Routine histological stains, such as Haematoxylin and Eosin (H and E) and trichrome stains, are used to illustrate changes in the shape, morphology and cytochemical architecture of the muscle fibres following denervation. Many fibres are seen as being angular in shape and granular in appearance. The granular fibres stain bluish with H and E and red with modified Gomori's trichrome, hence the name "ragged red fibres". Necrotic fibres tend to be pale staining liquefied or hyaline fibres and are frequently filled with phagocytes. Target fibres tend to be

made up of three distinct zones: a clear central zone which is devoid of oxidative enzyme activity, a densely staining intermediate zone with increased oxidative enzyme activity and a relatively normal peripheral zone of intermediate activity (Engel 1961). "Moth-eaten fibres" are recognized by their patchy staining with oxidative enzymes and their generally moth-eaten appearance. Fibre splitting is another common feature although this is also common in normal muscle at the myotendinous junction, hence care must be taken in interpreting pathological changes in this region. Fibre splitting is also apparent in normal muscle as a result of hypertrophy after some types of exercise. Coil fibres (or whorled fibres) are characterized by the disorientation of the longitudinal pattern of the myofibrils which results in a whorled appearance. They can also form giant fibres which are an aggregation of several smaller fibres (Dubowitz 1985).

At an ultrastructural level many changes occur in the myofibrils, mitochondria, sarcoplasmic reticulum, free ribosomes and the T-system. The myofibrils become irregular in shape, smaller in diameter and there is a decrease in the number of myofibrils contained within a fibre (Sunderland 1978, Jennekens 1982). The degree of myofibrillar atrophy is proportionate to, or slightly greater than, that of the muscle fibres (Stonnington and Engel 1973, Sunderland 1978). The mitochondria show changes in structure and position by becoming small and rounded, losing cristae, increasing in electron density, developing granules or vesicles and aligning along the axis of the myofibrils instead of encircling them (Jennekens 1982). After denervation, the T tubules tend to change in orientation from transverse to longitudinal (Gauthier and Dunn 1973, Libelius et al 1979) with

an associated increase in irregularity of the spatial arrangement (Gori 1972, Tomanck and Lund 1973). Rough endoplasmic reticulum and free ribosomes are not normally prominent in adult muscle but they are found after denervation (Gauthier and Schaeffer 1974). It is thought that the proliferation of these organelles is required for the synthesis of Ach receptor proteins and of enzymes involved in the degeneration of muscle fibres.

There is general agreement that the nuclei tend to be seen in clumps or rows, often in a more central position in the fibre as opposed to the peripheral location in normal muscle (Bowden and Gutmann 1944, Seddon 1975). The nuclei are thought to become larger and more oval early in the process of atrophy, returning to their original form once atrophy is well advanced (Sunderland and Ray 1950, Sunderland 1978). The number of nucleoli present tends to increase from the normal one or two, up to as many as four (Bowden and Gutmann 1944).

A sequence of events is initiated in the muscle fibres within a few days of transection of the nerve which supplies it. These events enhance the muscle fibres sensitivity to the effects of regenerating axons (Kuffler et al 1984). The resting membrane potential rises from about -75mV to about -60mV, approaching the threshold for action potential generation and is one of the factors which accounts for spontaneous fibrillation of denervated muscle (Slater and Harris 1988). Acetylcholine receptors appear in regions of the denervated muscle fibres not associated with the neuromuscular junction. This increases the sensitivity of the muscle fibres to acetylcholine, a condition which resembles that of embryonic muscle fibres (Diamond and Miledi 1962, Albaquerque and McIsaac 1969). The

Acetylcholine receptors are not evenly distributed on the surface of the muscle fibres and it is thought that the sites at which accumulations of these cells occur become sites for reinnervation (Frank et al 1975). There is evidence that these extrajunctional Ach receptors play a part in eliciting motor nerve sprouting (Jennekens 1982). Six to eight weeks after denervation these extrajunctional Ach receptors can no longer be found. N-CAM (nerve cell adhesion molecule) also appears on the surface of the muscle following denervation. It is normally concentrated primarily in the motor end plates but during development and following denervation there is rapid rise in the level present and in its distribution (Rieger et al 1985, Moore and Walsh 1986). N-CAM is thought to favour the formation of new synaptic contacts by regenerating axons (Moore and Walsh 1986, Slater and Harris 1988).

2. Reinnervation.

Neuromuscular transmission is usually restored within a day or two after the arrival of the regenerating axon. Regenerating motor axons often reinnervate denervated muscle fibres at the site of the original neuromuscular junction. This tendency appears to be related to the presence of an accumulation of N-CAM and of factors in the basal lamina that induce the growing axon tip to differentiate into a presynaptic terminal (Sanes et al 1978). However, new neuromuscular junctions can also form elsewhere on the surface of the muscle, particularly at the sites where extrajunctional Ach receptors have accumulated - see above.

Reinnervation is very effective if it occurs soon after the nerve has been injured, but becomes less so as the time between injury and the arrival of the

regenerating axons at the muscle increases (Gutmann and Young 1944, Seddon 1975). If the delay between denervation and reinnervation is too great then the proliferation of connective tissue interferes with the reinnervation process by blocking the Schwann tubes leading to the end plates (Bowden and Gutmann 1944, Seddon 1975). This may result in the formation of new end plates; however these tend to be less efficient than the original ones (Sunderland 1978). The degree of muscle fibre atrophy increases with the period of denervation. If the fibres are seriously atrophied this reduces the likelihood that the regenerating axons will re-establish continuity and therefore full maturation of the nerve fibre may be prevented (Sunderland 1978). The time between denervation and reinnervation is influenced by the distance between the lesion and the muscle, the type of injury sustained by the nerve and the method of repair (Sunderland 1978). The distance of the lesion from the muscle is the distance over which the nerve must regenerate before it can reform functional connections, obviously the greater the distance from the muscle, the longer the nerve will take to reach it. The nature of the injury also has an influence over the success of regeneration, with axonotmesis resulting in more rapid and accurate reinnervation than neurotmesis and repair (Sunderland 1978) - see also "Factors influencing the quality of motor recovery".

Effective reinnervation of the muscle is followed by a reversal of the effects of denervation. The resting potential returns to normal, extrajunctional sensitivity to acetylcholine is lost and with time the muscle fibres approximately regain their original dimensions (Slater and Harris 1988).

Reinnervated muscles show a tendency for type grouping of fast and slow

muscle fibres (Jaweed et al 1975). Myosin adenosine triphosphatase (ATPase) is a histochemical reaction which is used to differentiate type I and type II muscle fibres. The use of the enzyme provides a dependable technique for the identification of muscle fibre types, especially after denervation when the enzyme activities of the Krebs cycle and the glycolytic metabolism are greatly retarded (Drachman 1974). In a normal muscle the fibres belonging to different motor units are usually randomly distributed (Edstrom and Kugelberg 1968, Mayer and Doyle 1970, Kugelberg 1973 and 1979, Dubowitz 1985). When histochemical stains, such as ATPase, are applied this results in a mosaic like pattern of enzyme activity (Jennekens 1982). The mosaic pattern approximately conforms to a random distribution (Swash and Schwartz 1984). Following the reinnervation of muscles after the transection of their supplying nerve, the mosaic pattern tends to be replaced by distinct groups of fibres of the same histochemical type. This fibre type grouping was first described by Brooke and Engel (1966) and was also seen after the section and suture of a motor nerve by Karpati and Engel (1968c) and after cross reinnervation by Dubowitz (1967a) and Romanul and Van der Meulen (1967). The grouping of the motor units is the result of collateral sprouting of the regenerating nerve. Not all of the regenerating nerve fibres will reach the distal portion of the nerve and yet many if not all of the muscle fibres will be reinnervated (Jennekens 1982). The regenerating nerves tend to throw out collateral sprouts which synapse with neighbouring muscle fibres, consequently muscle fibres of a single motor unit are grouped together instead of being intermingled with fibres of another (Jennekens 1982)

The morphological changes seen in the muscle fibres, after denervation and reinnervation, have an impact on the level of recovery of function. Muscle weakness may be a consequence of muscle fibre atrophy and/or the proliferation of connective tissue. The inability of muscles to contract rapidly or to maintain prolonged activity may be a consequence of a change in the relative abundance of the fibre types present. The clinical importance of these morphological changes will be discussed more fully in chapter 10. It is important to establish whether these morphological changes in the muscle fibres are influenced in any way by the method of repair. Thus although the morphological changes seen in target muscles after denervation and reinnervation have been well documented in the literature, to date, no long term comparative study has been undertaken to assess these changes after different methods of injury and repair or to assess how these effects change at different time periods after operation.

6.4 Aims of the study

This study aims to answer the following questions - after injury and repair of the peripheral nerve:

1. What alterations occur in the wet weight of target muscles?
2. What alterations occur in the incidence of pathological features in muscle fibres within the target muscles?
3. What alterations occur in the size and shape of muscle fibres within the target muscles?
4. What changes occur in the distribution and relative proportion of type I and type II muscle fibres within the target muscles?

5. What changes occur in the connective tissue content of target muscles?

6. Do the different types of injury and repair have any effect on the afore mentioned changes in the morphological features of the target muscles?

7. Do the aforementioned changes in morphological features of the target muscles alter with respect to time after operation?

Materials and Methods

7.1 Surgical procedures

The surgical procedures were as previously described in chapter 3.1.

The rats were assessed, as described below, at periods of 50, 100, 200 and 300 days after operation.

7.2 Excision of Target Muscles.

The rats were anaesthetized by means of an intramuscular injection of a 1:1 mix of Hypnorm (Jansen Pharmaceuticals, UK, 0.5ml kg^{-1}) and Hypnovel (Midazolam Hydrochloride 5mg ml^{-1}, Roche Products Ltd., Welwyn Garden City, UK). In each of the rats, an incision was made over the iliac crest in order to expose biceps femoris. The muscle was detached from its iliac origin and deflected distally, thus exposing the transected and repaired sciatic nerve. The sciatic nerve was examined to ensure that the surgical repair was intact. The incision was extended distally, along the full length of the tibia, thus exposing the extensor retinaculum. This was transected in order to free the tendons associated with the peroneal muscles. Tibialis anterior was detached from its tibial origin and deflected proximally, in order to expose extensor digitorum longus (EDL) - figure 7.2.1. The tendons were transected as close to the myotendonous junction as possible and the target muscle (EDL) excised (figure 7.2.2).

The incision was extended dorsally around the ankle in order to expose tendo calcaneus. The tendon was transected, gastrocnemius deflected proximally thus

exposing the other target muscle soleus (figure 7.2.1). The tendons were transected as close to the myotendonous junction as possible and the target muscle excised (figure 7.2.2).

The right EDL and soleus muscles were excised in an identical manner to act as contralateral controls. The rats were then killed humanely.

7.3 Freezing of the Muscle.

Immediately after excision, all muscles were weighed and wrapped in foil until the preparations for the freezing process were complete. Each muscle was transected through the mid-belly and mounted cut face uppermost on a labelled cork disc. The tissue was supported on either side by gelatin, coated in Tissue Freezing Medium (Reichert-Jung, Cambridge Instruments) and snap frozen in dichlorodifluoromethane (Arcton 12, ICI) which was cooled to its melting point of approximately -150^{o}C in liquid nitrogen. The blocks were stored at -70^{o}C until required for sectioning.

7.4 Tissue Sectioning.

When required for sectioning, the tissue blocks were placed in a Frigocut 2800E Cryostat (Reichert-Jung, Cambridge Instruments), where they were left for a minimum of 10 minutes to allow the temperature to rise from the storage temperature of -70^{o}C to the cutting temperature of -20 to -25^{o}C. The muscles were serially sectioned once correct orientation of the muscle fibres had been assessed, after staining the sections with toluidine blue on a test slide. The 10μm sections were mounted on clean glass slides, allowed to air dry and stored at -70^{o}C until required for staining.

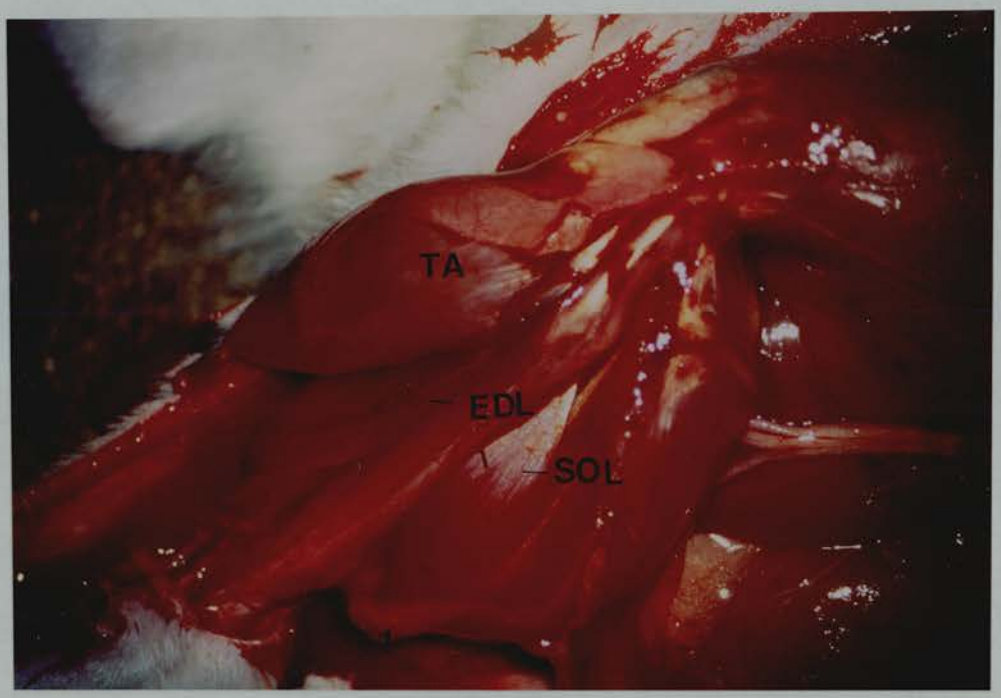

Figure 7.2.1 - Position of the target muscles, extensor digitorum longus (EDL) and soleus (Sol), in the lower hind limb of the rat (TA = the deflected tibialis anterior).

Figure 7.2.2 - Excised target muscles, EDL and soleus.

7.5 Histological and Histochemical stains.

The following histological and histochemical staining techniques were routinely applied to the sections from all experimental and contralateral control groups:-

(a) Toluidine blue

Toluidine blue stains the tissue components varying shades of blue and is used to check the correct orientation and preservation of the block during sectioning. In cases where problems arose with the haematoxylin and eosin stain, it was used to assess muscle fibre architecture for features indicating denervation and/or reinnervation.

(b) Haematoxylin and eosin (H and E)

Haematoxylin and eosin stains the nuclei and basophilic material blue-black and the cytoplasm pink. It was used to assess muscle fibre architecture, as stated above.

(c) Modified Gomori's trichrome

Modified Gomori's trichrome stains the muscle fibres blue-green, the intermyofibrillar tubules, mitochondria and membrane bound fat droplets bright red and endomysial connective tissue stains green. Nuclei appear reddish-green. It was used to highlight 'ragged red' fibres, which stand out as bright red against the blue-green of the other muscle fibres.

(d) Masson's trichrome

Masson's trichrome stains the muscle fibres red-brown, the nuclei blue-black

and the connective tissue bright green. It was used to assess muscle fibre architecture and the proliferation of connective tissue.

(e) Nicotinamideadenine dinucleotide tetrazolium reductase(NADH-TR)

NADH-TR stains the mitochondria, sarcoplasmic reticulum and T tubules dark blue. Type I fibres give a strong reaction whilst type II fibres give a much weaker reaction. It can be used for fibre typing and to assess the distortion of myofibrillar material.

(f) Succinate dehydrogenase (SDH)

SDH stains the intermyofibrillar and subsarcolemmal mitochondria blue. Type I fibres give a strong reaction whilst type II fibres give a much weaker reaction. It can be used for fibre typing and to show an excess of mitochondria.

(g) Myosin Adenosine Triphosphatase (ATPase) - pH 10.2, 4.6 and 4.35

ATPase stains the muscle fibres light, dark or intermediate depending on the preincubation medium. At pH 10.2 type I fibres are light and all type II fibres are dark, at pH 4.6 type IIa fibres are light and all other fibre types are dark and at pH 4.35 type I fibres are dark and type IIa and b fibres are light.

Although all of the above stains were routinely applied to the sections from all experimental and contralateral control groups, not all were subsequently used in the assessment of the muscle. Modified Gomori's trichrome was applied to all sections and despite plenty of evidence to indicate the presence of necrotic fibres, not a single ragged red fibre was seen. The other signs of the presence of necrotic fibres indicate that the absence of ragged red fibres was due to a shortcoming of

the stain and not the absence of such fibres. The use of NADH-TR and SDH reactions produced very variable results. The inconsistencies in the staining produced with these reactions was felt to be too great to allow their inclusion in the study.

Details of the staining techniques are given in appendix 3.

7.6 Qualitative Assessment of the muscles.

1. Incidence of muscle fires with pathological features.

Using sections stained with haematoxylin and eosin, a qualitative assessment was made of the incidence of muscle fibres showing pathological features. The muscle sections were scanned and a total of 500 fibres were assessed for the presence of internal nuclei, angular shape, granular appearance, 'moth eaten' appearance, circular or whorled fibres and split fibres. Each pathological feature was graded as either - (not present), * (< 15 fibres), ** (15 - 30 fibres), *** (30 - 50 fibres) or **** (> 50 fibres). Modified Gomori's trichrome was used to illustrate the presence or absence of ragged red fibres.

2. Histochemical fibre types.

Using sections stained with ATPase, a qualitative assessment was made of different histochemical fibre types. The relative abundance of each fibre type present was assessed and a note made of fibre type predominance. The distribution of fibre types was assessed and a note made of whether it was a random distribution in the classic mosaic pattern or whether fibre type grouping was evident. Fibre type grouping was said to be evident if two fibres were enclosed on

all sides by fibres of the same histochemical type; the enclosed fibre theory of Jennekens et al (1971). The predominance of one fibre type was taken into consideration when assessing fibre type grouping.

3. Size range of muscle fibres and fascicles.

The size range of the muscle fibres and the fascicles were noted and recorded as either normal or variable. If recorded as normal then the size of the fibres and fascicles fell in the normal range of sizes expected whereas those recorded as variable covered a larger range than in normal muscle.

7.7 Quantitative Assessment of the muscles.

1. Measurement of muscle fibres.

A Magiscan morphometric analysis system (Joyce-Loebl, UK Ltd.) was used to assess the size and shape of the muscle fibres on sections stained for ATPase pH 10.2 and pH 4.35. In accordance with the recommendation of Swash and Schwartz (1984) all fibres contained within an arbitrary number, not less than 5, different microscopic fields were measured. In the current study a total of 200 muscle fibres was assessed for each specimen, as opposed to the minimum of 100 fibres recommended by Swash and Schwartz (1984). The proportion of type I and type II fibres present was calculated. The 200 fibres were measured and the maximum fibre diameter, minimum fibre diameter, cross sectional area, perimeter and form factor were calculated. The best measure of fibre size is the minimum fibre diameter, as this measurement overcomes the distortion which occurs when a muscle fibre is cut obliquely (Dubowitz 1985). The maximum diameter was defined

as being the distance between the two points on the boundary which are furthest apart. The minimum fibre diameter was defined as being the greatest distance between the opposite sides of the narrowest aspect of the muscle fibre (Aherne and Dunhill 1982). The area was derived purely from the boundary, no account was taken of any holes which occurred within the fibre. The perimeter is the sum of the distances between the mid point of the boundary vectors. The shape of the muscle fibre was calculated as the form factor which is four times the area times pi divided by the perimeter squared, i.e.

$$Form\ factor = \frac{4 \times a \times \Pi}{p^2}$$

This value equals one for a perfect circle.

The results were compared to assess any differences which occur in the size and shape of the reinnervated muscle fibres at the specified time periods after each of the methods of injury and repair.

2. Connective tissue content of the muscle.

The connective tissue content of the muscle was measured quantitatively using a point counting technique (Aherne and Dunhill 1982). A 100 point graticule was placed in the eye piece of the microscope, which resulted in the image of the grid overlying the image of the muscle cross section. A count was made of the number of grid intersections which fell on connective tissue. Sections used in the point counting process were stained with Masson's Trichrome which stains the connective tissue a distinctive green colour. All point counts were made using a x40 magnification.

A trial set of counts was initially undertaken to enable the estimation of the minimum number of intercept points which must be counted, to ensure an acceptable relative standard error (RSE). This was calculated using a formula introduced by Hally (1964),

i.e.

$$RSE = \sqrt{\left(\frac{1-Vv}{n}\right)}$$

where:

n = the number of points falling on connective tissue

N = The total number of points falling on the muscle.

$$Vv = \frac{n}{N}$$

The mean RSE in calculating the volume percentage of connective tissue in EDL and soleus was 4.97% for experimental muscles and 5.64% for contralateral control muscles, with an overall mean of 5.31%. To ensure this RSE of approximately 5% a total of 2500 points were observed for each specimen.

7.8 Statistical Analysis.

The null hypothesis.

In the current study the null hypothesis states that there is no real difference in the size, shape and cytochemical architecture of the target muscle fibres after each type of peripheral nerve injury and repair.

Statistical analysis.

The distribution of the data were assessed for normality as previously described in chapter 3.8. The data was subsequently analyzed using ANOVA, the Student's t-test or the Mann Whitney U test as appropriate - see chapter 3.8.

Statistical Significance.

The statistical significance was assessed at the 5% level. The results are summarized in tables 8.1.1- 8.1.4, 8.3.2 - 8.3.5, 8.3.7, 8.3.9 - 8.3.11 and 8.3.13 to 8.3.16

7.9 Steps taken to avoid systematic errors.

Inter-animal variation was kept to a minimum by ensuring that animal species, sex and weight were consistent among groups. All samples of EDL and soleus were taken from the mid-belly of the muscle and sectioned in the same way. This ought to ensure that any changes seen in the muscle fibre architecture and in the amount of connective tissue present, were real and not due to variations normally seen along the length of the muscle. This is particularly true in the case of connective tissue since, as a rule, there is a greater proportion of connective tissue near the myotendonous junction than in the mid-belly of the muscle (Kennedy 1987). Similarly, fibre splitting is a much more common occurrence near the myotendonous junction than elsewhere in the muscle. If care is not taken then such normal anatomy could be interpreted as pathological change due to denervation/reinnervation.

Many studies which have been undertaken to provide a quantitative analysis

of muscle fibre size and shape variation, have relied on sections stained for NADH. The reason behind the preference for this stain appears to lie in the fact that the NADH stain results in a lesser degree of tissue shrinkage than many other stains. Kennedy (1987) showed that the ATPase stain resulted in muscle fibre shrinkage of 1.6% when compared to the NADH stain. Despite this apparent shrinkage the majority of the quantitative analysis in the current study was undertaken on sections stained for ATPase. The reason for this was that the ATPase stain produced far more reliable and consistent staining of the muscle fibres than many of the other histochemical stains. The ATPase stain also had the added advantage that the resultant black and white image provided the absolute contrast required for the image analysis system. The shrinkage which this stain caused must be taken into consideration when comparing the results of the current study with others in the literature which have used different stains. In this comparative study, it is a constant error affecting all groups equally and hence can be ignored.

In the quantitative analysis of muscle fibre size, shape and type, a total of 200 fibres was assessed for each specimen. Measuring 200 fibres is ordinarily sufficient to give consistent and reproducible results (Dubowitz 1985). A count was made of all the fibres in 5-6 randomly placed fields along the long axis of the muscle section, until a total of 200 fibres had been counted. An initial assessment was made to find the number of consecutive fields which lay along the long axis of the section. A calculation was then made to assess whether it was necessary to count every second, third, fourth, etc. field in order to ensure that the entire length of the muscle was assessed. The initial field in the series was chosen at random by picking

a number out of a box (if every alternate field was to be counted then numbers 1 and 2 were put in the box, if every third field was to be counted then numbers 1, 2 and 3 were entered in the box etc.).

All observations in the point counting process were made at x40 magnification. It is generally assumed that the estimation of volume fraction and surface area is independent of the magnification used in gaining the counts (Aherne and Dunhill 1982). It has now been shown by some authors that an increase in magnification does lead to an increase in surface area estimation (Gehr et al 1976), however Olah (1976) concluded that magnification had no influence on volume fraction calculations. Despite this finding it was decided to avoid any possible additional errors in the current study by using a constant magnification of x40 for all observations. Since this is a comparative study, with a constant magnification, any possible errors associated with the magnification can safely be ignored, however caution must be used when comparing the results with those of similar studies.

RESULTS

All rats showed progressive signs of recovery up to 300 days after operation, by which time the toe spreading reflex (Young 1942) could be elicited in 95% of experimental animals. The return of this reflex is said to reflect good motor reinnervation by the sciatic nerve (Myles et al 1992). As in the previous study (chapters 2 to 5) the 5% of animals who failed to regain their toe spreading reflex on the repaired side were found to have acquired fixed deformities of the ankle joint and this prevented any movement of the foot. This occurred in 3 animals whose nerves had been transected and then repaired by means of the muscle graft and in one which received a nerve graft.

8.1 Alterations in the wet weight of muscle after injury and repair.

The experimental and contralateral control muscles were weighed, to within 0.01g, immediately following excision. In order to allow direct comparison of the weight loss of experimental muscles in animals of different weights (i.e. animals at different time periods after operation), the wet muscle weights were standardized. The weights were standardized by multiplying the wet muscle weight by 1000 and dividing by the weight of the animal.

Table 8.1.1 (A) and figure 8.1.1 (A- H) illustrate the standardized wet weight of both the experimental and contralateral control muscles with respect to time and the method of injury and repair. The graphs clearly show that the standardized wet weight of the experimental muscle is consistently less than that of the contralateral control muscles. This is true at all time periods after operation and after all types of

injury and repair. The difference in weight between the experimental and contralateral control muscles was most marked at 50 days after operation, with the difference reaching statistical significance in all but the nerve crush EDL group - see table 8.1.1(B). Indeed in 50% of the experimental groups the statistical significance reached the 0.1% level on comparison with the contralateral controls. The difference in weight between the experimental and contralateral control muscles decreased with time so that by 300 days after operation there was no significant difference between the two values. The only exception to this rule involved animals whose sciatic nerve had been repaired by means of the insertion of a muscle graft. By 300 days after operation the difference in weight between the experimental and contralateral control muscles in this group was less marked than at any other time period; however it did still reach statistical significance ($p < 0.01$).

In order to assess the changes seen in the weight of the muscles with respect to time and experimental method, the weight of the experimental muscle was expressed as a percentage of the weight of the contralateral control muscle, illustrated in figure 8.1.2. This allowed the direct comparison of the level of recovery of the experimental muscle (in terms of muscle weight) as compared to the contralateral control. At 50 days after a nerve crush injury the EDL muscle showed a significantly greater level of recovery of muscle weight than after any injury which involves the transection of the nerve ($p < 0.05$ in each case) - see table 8.1.2. This was also true for the soleus muscle where significance reached the 5% level on comparing the results after a nerve crush with the muscle graft and nerve to nerve suture results and reached the 0.1% level on comparison with the results

Repair	Muscle	Weight of muscle (g)			
		50days	100day	200day	300day
Muscle Graft	Control EDL	0.46	0.48	0.42	0.42
	Experimental EDL	0.23	0.31	0.32	0.37
	Control Soleus	0.46	0.46	0.37	0.35
	Experimental Soleus	0.27	0.24	0.23	0.29
Nerve Crush	Control EDL	0.43	0.45	0.45	0.43
	Experimental EDL	0.37	0.40	0.43	0.40
	Control Soleus	0.42	0.43	0.42	0.40
	Experimental Soleus	0.37	0.36	0.38	0.38
Nerve Graft	Control EDL	0.44	0.46	0.47	0.42
	Experimental EDL	0.28	0.38	0.41	0.38
	Control Soleus	0.42	0.40	0.42	0.37
	Experimental Soleus	0.22	0.32	0.34	0.30
N-N Suture	Control EDL	0.42	0.45	0.42	0.41
	Experimental EDL	0.25	0.35	0.42	0.36
	Control Soleus	0.40	0.43	0.42	0.36
	Experimental Soleus	0.26	0.28	0.37	0.30

Table 8.1.1 (A) - The standardized wet weight (g) of the experimental and **contralateral control** soleus and EDL after each type of injury and repair and at each time period after operation.

Repair	Muscle	Significance levels			
		50days	100days	200days	300days
Muscle Graft	EDL	$p < 0.001$	$p < 0.01$	$p < 0.01$	$p < 0.001$
	Soleus	$p < 0.05$	$p < 0.001$	$p < 0.01$	$p < 0.01$
Nerve Crush	EDL	N.S.	$p < 0.001$	$p < 0.01$	N.S.
	Soleus	$p < 0.05$	$p < 0.001$	$p < 0.05$	N.S.
Nerve Graft	EDL	$p < 0.001$	$p < 0.05$	$p < 0.01$	N.S.
	Soleus	$p < 0.001$	N.S.	$p < 0.05$	N.S.
N-N Suture	EDL	$p < 0.01$	$p < 0.01$	N.S.	N.S.
	Soleus	$p < 0.001$	$p < 0.01$	$p < 0.01$	N.S.

Table 8.1.1 (B) - The significance values for the comparison of the standardized wet weight (g) of the experimental and contralateral control soleus and EDL at each time period after operation. (N.S. = no significant difference)

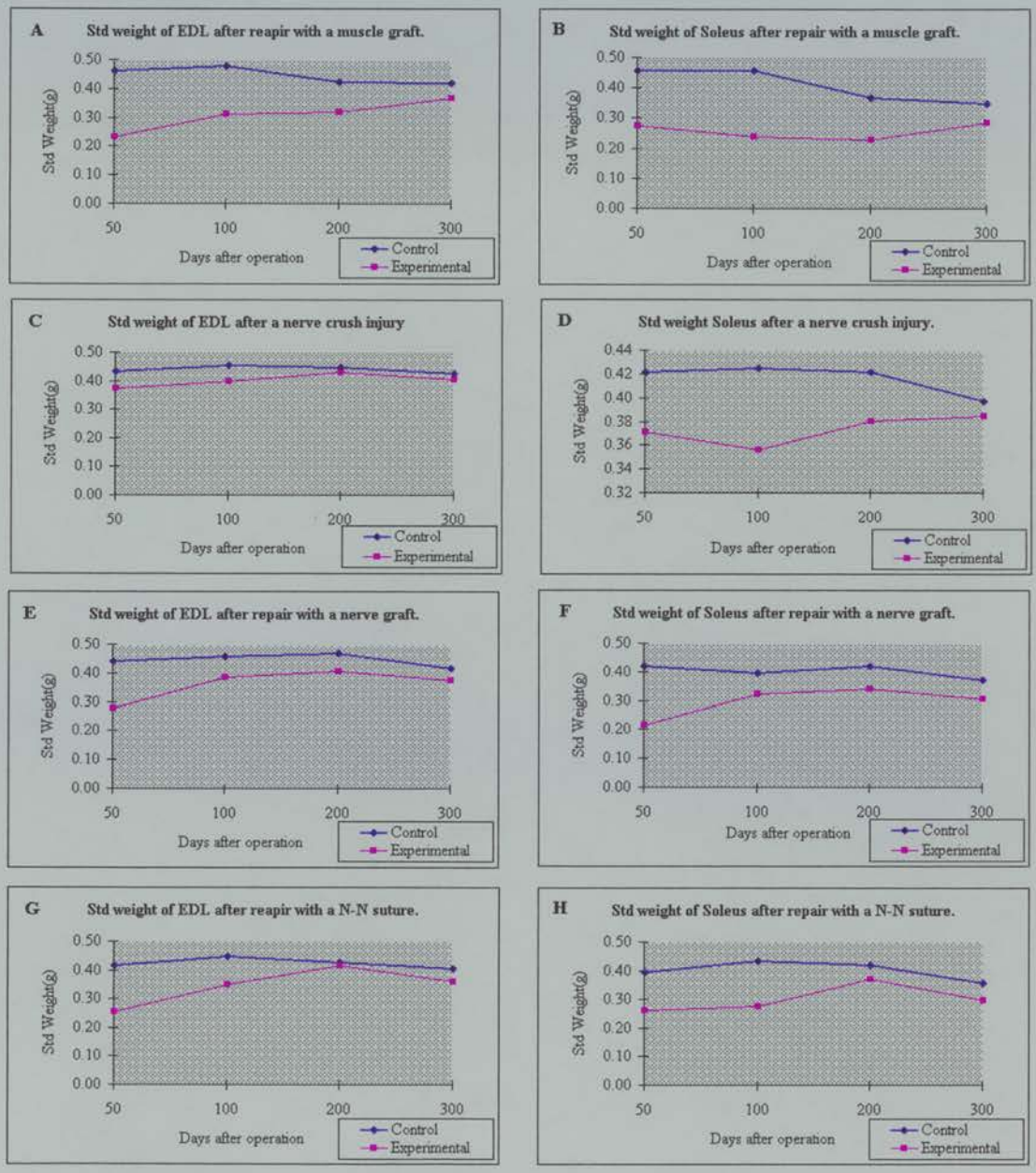

Figure 8.1.1 (A - H) - The standardized wet weight (std weight) of the experimental and contralateral control muscles at each at each of the time periods after operation. The changes in the wet weight of EDL and soleus after the repair of the sciatic nerve with a muscle graft are represented in graphs (A) and (B), after a nerve crush injury in graphs (C) and (D), after repair with a nerve graft in graphs (E) and (F) and after a direct epineurial suture in graphs (G) and (H).

Repair	Muscle	Exp. muscle weight as % of c.control			
		50days	100days	200days	300days
Muscle Graft	EDL	50.00	64.55	76.19	88.09
	Soleus	58.69	52.17	62.16	82.86
Nerve Crush	EDL	86.04	88.89	95.56	93.02
	Soleus	88.09	83.72	90.48	95.00
Nerve Graft	EDL	68.63	82.60	87.23	90.47
	Soleus	52.38	80.00	80.95	81.08
N-N Suture	EDL	59.52	77.78	100.00	87.80
	Soleus	65.00	65.11	88.09	83.33

Table 8.1.2 (A) - The standardized wet weight of the experimental muscles expressed as a percentage of the contralateral control muscle weight after each type of injury and repair and at each time period after operation.

(A)

Repair	50 Days EDL			
	Muscle Graft	Nerve Crush	Nerve Graft	N-N Suture
Muscle Graft	*	*	*	*
Nerve Crush	$p < 0.05$	*	*	*
Nerve Graft	$p < 0.05$	$p < 0.05$	*	*
N-N Suture	N.S.	$p < 0.05$	N.S.	*

(B)

Repair	100 Days EDL			
	Muscle Graft	Nerve Crush	Nerve Graft	N-N Suture
Muscle Graft	*	*	*	*
Nerve Crush	$p < 0.01$	*	*	*
Nerve Graft	N.S.	N.S.	*	*
N-N Suture	N.S.	N.S.	N.S.	*

(C)

Repair	200 Days EDL			
	Muscle Graft	Nerve Crush	Nerve Graft	N-N Suture
Muscle Graft	*	*	*	*
Nerve Crush	$p < 0.01$	*	*	*
Nerve Graft	$p < 0.05$	$p < 0.01$	*	*
N-N Suture	$p < 0.01$	N.S.	$p < 0.01$	*

(D)

Repair	300 Days EDL			
	Muscle Graft	Nerve Crush	Nerve Graft	N-N Suture
Muscle Graft	*	*	*	*
Nerve Crush	$p < 0.05$	*	*	*
Nerve Graft	N.S.	N.S.	*	*
N-N Suture	N.S.	N.S.	N.S.	*

Table 8.1.2 (B) - The significance values for the comparison of the wet weight of experimental **EDL** (expressed as a percentage of the contralateral control muscle weight) after each type of injury and repair. (A) 50 days after operation, (B) 100 days after operation, (C) 200 days after operation and (D) 300 days after operation. (N.S. = no significant difference).

(E)

Repair	50 Days Soleus			
	Muscle Graft	Nerve Crush	Nerve Graft	N-N Suture
Muscle Graft	*	*	*	*
Nerve Crush	N.S.	*	*	*
Nerve Graft	N.S.	$p < 0.001$	*	*
N-N Suture	N.S.	$p < 0.05$	$p < 0.01$	*

(F)

Repair	100 Days Soleus			
	Muscle Graft	Nerve Crush	Nerve Graft	N-N Suture
Muscle Graft	*	*	*	*
Nerve Crush	$p < 0.01$	*	*	*
Nerve Graft	N.S.	N.S.	*	*
N-N Suture	N.S.	$p < 0.01$	N.S.	*

(G)

Repair	200 Days Soleus			
	Muscle Graft	Nerve Crush	Nerve Graft	N-N Suture
Muscle Graft	*	*	*	*
Nerve Crush	$p < 0.001$	*	*	*
Nerve Graft	$p < 0.05$	N.S.	*	*
N-N Suture	$p < 0.01$	N.S.	N.S.	*

(H)

Repair	300 Days Soleus			
	Muscle Graft	Nerve Crush	Nerve Graft	N-N Suture
Muscle Graft	*	*	*	*
Nerve Crush	$p < 0.05$	*	*	*
Nerve Graft	N.S.	N.S.	*	*
N-N Suture	N.S.	N.S.	N.S.	*

Table 8.1.2 (C) - The significance values for the comparison of the wet weight of experimental **soleus** (expressed as a percentage of the contralateral control muscle weight) after each type of injury and repair. (E) 50 days after operation, (F) 100 days after operation, (G) 200 days after operation and (H) 300 days after operation. (N.S. = no significant difference).

after a nerve graft. At 100 days after operation in both the soleus and the EDL, the muscle graft group showed a significantly lower level of recovery of muscle weight than the nerve crush group ($p < 0.01$). In the EDL muscle there was no significant difference in the level of recovery between the other methods of repair, whereas in the soleus muscle the nerve crush injury also resulted in a significantly greater level of recovery than after the nerve to nerve suture ($p < 0.01$) - see table 8.1.2. At 200 days after repair by means of a muscle graft both the soleus and the EDL muscles showed a significantly lower level of recovery of muscle weight than after any other form of injury and repair. This difference was significant at the 5% level on comparing the results after repair with a muscle graft to those after a nerve graft and at the 1% level on comparing the results after repair with a muscle graft to those after a nerve to nerve suture or nerve crush. In the EDL there was also a significant difference in the level of recovery of muscle weight between those animals repaired with a nerve graft and those repaired with a nerve to nerve suture or injured by a nerve crush ($p < 0.01$ in each case) - see table 8.1.2. This was not the case in the soleus muscle. At 300 days after operation, in both the soleus and EDL, the muscle graft group showed a significantly lower level of recovery of muscle weight than after a nerve crush injury ($p < 0.05$). There was no significant difference between any of the other methods of injury and repair in either of the experimental muscles at 300 days after operation - see table 8.1.2.

Thus repair by means of a muscle graft consistently resulted in a significantly lower level of recovery of muscle weight than after a nerve crush injury. There was no other consistent difference between the different methods of injury and repair.

The soleus muscle tended to show a lower level of recovery of muscle weight than the EDL muscle. This difference reached statistical significance 50 days after the insertion of a nerve graft ($p < 0.05$), 100 and 200 days after the insertion of a muscle graft ($p < 0.01$ in each case) and 100 and 200 days after repair by nerve to nerve suture ($p < 0.05$ in each case) - see table 8.1.3. By 300 days after injury and repair there was no significant difference between the recovery of muscle weight in the soleus and the EDL.

When considering the different methods of injury and repair with respect to time, the EDL muscle consistently showed a significant increase in the level of recovery of muscle weight from 50 to 300 days after operation - see figure 8.1.2 (graphs A - D) and table 8.1.4. This increase in muscle weight was significant at the 5% level in the nerve graft and nerve to nerve suture groups and at the 0.1% level in the muscle graft group, however it failed to reach statistical significance in the nerve crush group. The soleus muscle showed a similar significant increase in the level of recovery from 50 to 300 days after repair by means of a nerve to nerve suture ($p < 0.001$) or a nerve graft ($p < 0.05$). There was a similar increase in the level of recovery over the same time period after a nerve crush injury or repair by means of a muscle graft however this did not reach statistical significance.

8.2 Qualitative assessment of muscles.

1. Incidence of muscle fibres with pathological features.

Table 8.2.1 illustrates the incidence of muscle fibres with pathological features after each type of injury and repair at each of the time periods after operation. Muscle fibres with pathological features were present both in experimental and contralateral control muscles. The number of fibres in

Repair	Days after operation			
	50	100	200	300
Muscle Graft	N.S.	$p < 0.01$	$p < 0.01$	N.S.
Nerve Crush	N.S.	N.S.	N.S.	N.S.
Nerve Graft	$p < 0.05$	N.S.	N.S.	N.S.
N-N Suture	N.S.	$p < 0.05$	$p < 0.05$	N.S.

Table 8.1.3 - The significance values for the comparison of the wet muscle weight of the experimental EDL and soleus (expressed as a percentage of the contralateral control muscle weight) after each method of injury and repair and at each time period after operation. (N.S. = no significant difference).

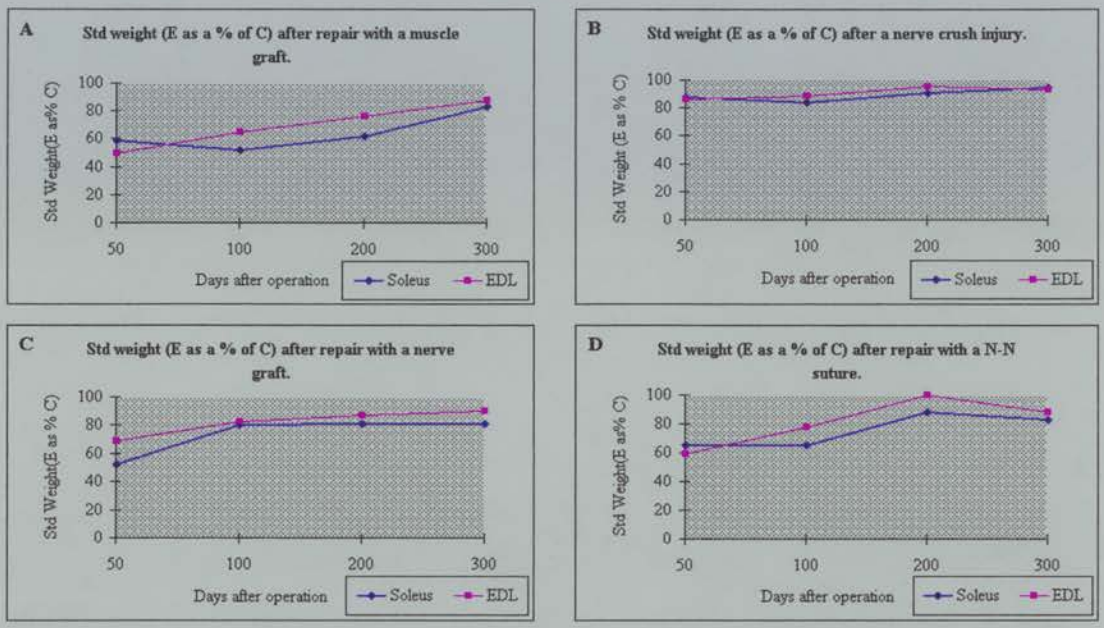

Figure 8.1.2 (A - D) - The standardized wet weight (std weight) of the experimental muscles (expressed as a percentage of the contralateral control muscle weight) at each time period after (A) repair with a muscle graft, (B) a nerve crush injury, (C) repair with a nerve graft and (D) repair with a direct epineurial suture.

(A)

Repair	50 Days EDL			
	Muscle Graft	Nerve Crush	Nerve Graft	N-N Suture
Muscle Graft	*	*	*	*
Nerve Crush	N.S.	*	*	*
Nerve Graft	$p < 0.05$	$p < 0.01$	*	*
N-N Suture	$p < 0.001$	$p < 0.05$	$p < 0.05$	*

(B)

Repair	100 Days EDL			
	Muscle Graft	Nerve Crush	Nerve Graft	N-N Suture
Muscle Graft	*	*	*	*
Nerve Crush	N.S.	*	*	*
Nerve Graft	N.S.	$p < 0.01$	*	*
N-N Suture	N.S.	$p < 0.05$	N.S.	*

(C)

Repair	200 Days EDL			
	Muscle Graft	Nerve Crush	Nerve Graft	N-N Suture
Muscle Graft	*	*	*	*
Nerve Crush	$p < 0.01$	*	*	*
Nerve Graft	$p < 0.01$	N.S.	*	*
N-N Suture	$p < 0.05$	N.S.	N.S.	*

(D)

Repair	300 Days EDL			
	Muscle Graft	Nerve Crush	Nerve Graft	N-N Suture
Muscle Graft	*	*	*	*
Nerve Crush	$p < 0.05$	*	*	*
Nerve Graft	$p < 0.01$	$p < 0.01$	*	*
N-N Suture	$p < 0.05$	N.S.	N.S.	*

Table 8.1.4 (A) - The significance values for the comparison of the wet weight of experimental **EDL** (expressed as a percentage of contralateral control muscle weight) after each type of injury and repair. (A) 50 days after operation, (B) 100 days after operation, (C) 200 days after operation and (D) 300 days after operation. (N.S. = no significant difference).

(E)

Repair	50 Days Soleus			
	Muscle Graft	Nerve Crush	Nerve Graft	N-N Suture
Muscle Graft	*	*	*	*
Nerve Crush	N.S.	*	*	*
Nerve Graft	N.S.	N.S.	*	*
N-N Suture	N.S.	$p < 0.01$	$p < 0.05$	*

(F)

Repair	100 Days Soleus			
	Muscle Graft	Nerve Crush	Nerve Graft	N-N Suture
Muscle Graft	*	*	*	*
Nerve Crush	N.S.	*	*	*
Nerve Graft	N.S.	N.S.	*	*
N-N Suture	N.S.	$p < 0.05$	N.S.	*

(G)

Repair	200 Days Soleus			
	Muscle Graft	Nerve Crush	Nerve Graft	N-N Suture
Muscle Graft	*	*	*	*
Nerve Crush	N.S.	*	*	*
Nerve Graft	$p < 0.01$	N.S.	*	*
N-N Suture	$p < 0.05$	N.S.	N.S.	*

(H)

Repair	300 Days Soleus			
	Muscle Graft	Nerve Crush	Nerve Graft	N-N Suture
Muscle Graft	*	*	*	*
Nerve Crush	N.S.	*	*	*
Nerve Graft	$p < 0.05$	$p < 0.01$	*	*
N-N Suture	$p < 0.001$	$p < 0.05$	N.S.	*

Table 8.1.4 (B) - The significance values for the comparison of the wet weight of experimental **soleus** (expressed as a percentage of contralateral control muscle weight) after each type of injury and repair. (E) 50 days after operation, (F) 100 days after operation, (G) 200 days after operation and (H) 300 days after operation. (N.S. = no significant difference).

Repair	Muscle	Internal nuclei	Angular fibres	Granular fibres	Necrotic fibres	Circular fibres	Split fibres
50 days							
Nerve Crush	EDL (E)	*	*	*	*	-	**
	EDL (C)	-	-	-	-	-	-
	Soleus (E)	**	*	*	*	-	**
	Soleus (C)	*	*	*	*	-	*
N-N Suture	EDL (E)	***	****	****	***	*	***
	EDL (C)	-	-	-	-	-	**
	Soleus (E)	**	**	**	*	*	***
	Soleus (C)	**	*	*	*	-	**
Nerve Graft	EDL (E)	***	*	*	*	-	*
	EDL (C)	-	-	-	-	-	-
	Soleus (E)	*	**	**	*	-	*
	Soleus (C)	-	-	-	-	-	*
Muscle Graft	EDL (E)	**	***	***	**	-	***
	EDL (C)	-	-	-	-	-	-
	Soleus (E)	**	***	***	***	-	****
	Soleus (C)	-	-	-	-	-	-
100 days							
Nerve Crush	EDL (E)	*	**	**	-	-	**
	EDL (C)	-	*	*	-	-	-
	Soleus (E)	*	*	*	-	-	***
	Soleus (C)	*	*	-	-	-	*
N-N Suture	EDL (E)	***	***	***	**	-	***
	EDL (C)	-	*	*	-	-	**
	Soleus (E)	**	***	***	***	-	**
	Soleus (C)	-	-	-	-	-	**
Nerve Graft	EDL (E)	**	***	***	***	-	*
	EDL (C)	-	*	*	-	-	-
	Soleus (E)	**	****	***	**	-	***
	Soleus (C)	*	-	-	*	-	**
Muscle Graft	EDL (E)	***	**	**	*	-	*
	EDL (C)	-	*	*	-	-	*
	Soleus (E)	**	***	***	***	*	****
	Soleus (C)	**	*	*	*	**	***

Table 8.2.1 - A qualitative assessment of the presence of muscle fibres with pathological features in the experimental (E) and contralateral control (C) EDL and soleus. (- absent, * < 5 fibres, ** 5 - 15 fibres, *** 15 -30 fibres, **** > 30 fibres)

Repair	Muscle	Internal nuclei	Angular fibres	Granular fibres	Necrotic fibres	Circular fibres	Split fibres
200 days							
Nerve Crush	EDL (E)	**	**	**	*	-	**
	EDL (C)	-	-	-	-	-	-
	Soleus (E)	*	*	*	*	-	*
	Soleus (C)	*	-	-	-	-	*
N-N Suture	EDL (E)	**	**	**	**	-	**
	EDL (C)	-	-	-	-	-	-
	Soleus (E)	**	**	**	**	-	**
	Soleus (C)	*	-	-	*	*	*
Nerve Graft	EDL (E)	*	**	**	**	-	**
	EDL (C)	-	-	-	-	-	-
	Soleus (E)	**	**	**	**	**	**
	Soleus (C)	*	-	-	-	-	-
Muscle Graft	EDL (E)	**	**	**	**	-	**
	EDL (C)	-	-	-	-	-	-
	Soleus (E)	***	**	**	**	*	**
	Soleus (C)	*	-	-	-	*	*
300 days							
Nerve Crush	EDL (E)	*	-	-	-	-	**
	EDL (C)	-	-	-	-	-	-
	Soleus (E)	**	*	*	*	*	**
	Soleus (C)	*	*	-	*	*	**
N-N Suture	EDL (E)	**	*	*	*	-	**
	EDL (C)	-	-	-	-	-	-
	Soleus (E)	**	**	*	*	-	**
	Soleus (C)	*	*	-	-	-	**
Nerve Graft	EDL (E)	***	**	*	-	-	*
	EDL (C)	*	-	-	-	-	*
	Soleus (E)	***	**	**	**	-	***
	Soleus (C)	*	-	-	-	-	-
Muscle Graft	EDL (E)	***	**	**	**	*	***
	EDL (C)	*	-	-	-	-	-
	Soleus (E)	***	**	**	*	-	***
	Soleus (C)	**	*	*	*	*	**

Table 8.2.1 (continued) - A qualitative assessment of the presence of muscle fibres with pathological features in the experimental (E) and contralateral control (C) EDL and soleus. (- absent, * < 5 fibres, ** 5 - 15 fibres, *** 15 -30 fibres, **** > 30 fibres)

experimental muscles which were angular, granular, necrotic, split and/or had migrating nuclei was consistently greater than or equal to the number of such fibres in contralateral control muscles. The only pathological feature which did not fit in with this rule was the presence of circular or whorled fibres. The number of circular fibres present was greater in the experimental muscles in three cases, greater in contralateral control muscles in three cases and equal in all other cases. There was no consistency between the number of circular fibres present and the type of injury and repair. There was no consistency between the number of pathological features present and the time period which had elapsed since the injury and repair. Figure 8.2.1 shows normal muscle fibre architecture while figures 8.2.2 to 8.2.7 show the common pathological features of denervated/reinnervated muscle

In both the experimental and contralateral control muscles, split fibres and migrating nuclei were the most frequently occurring pathological features. Fewer than 3% of muscle fibres illustrated the presence of internal nuclei in the contralateral control muscles, with the exception of the soleus muscle 50 day nerve graft group and 100 day and 200 day muscle graft groups. In each of these cases the number of migrating nuclei was in the 3 to 6 % range. In most of the experimental muscles more than 3% of fibres had internal nuclei, the range was 3% to 10 %. The exceptions to this finding included the EDL muscle 50, 100 and 300 days after a nerve crush injury and 200 days after nerve graft insertion and the soleus muscle 100 and 200 days after crush injury and 50 days after nerve graft insertion. It is notable that of the experimental muscles in which less than 3% of fibres showed internal nuclear migration, 5 out of 7 had undergone the less severe

injury of a nerve crush rather than transection. The splitting of muscle fibres was found to occur in less than 6% of all contralateral control muscles and greater than 3% of all experimental muscles, with a range of 3 to >10 %. The highest incidence of fibre splitting was found in the soleus muscle 50 and 100 days after the insertion of a muscle graft.

After a nerve crush injury there were consistently fewer fibres with pathological features present in the muscles than after any of the injuries which involved the transection of the nerve. After the repair of a transected nerve using a muscle graft there was generally a greater number of fibres with pathological features than after a nerve to nerve suture which in turn resulted in a greater number than after a nerve graft. Quantitative analysis of these features would allow a more accurate assessment to be made of the consequences of each method of repair implemented. The relative proportion of fibres in EDL with pathological features was consistently less than or equal to the number present in soleus. This was true both for experimental and contralateral control muscles.

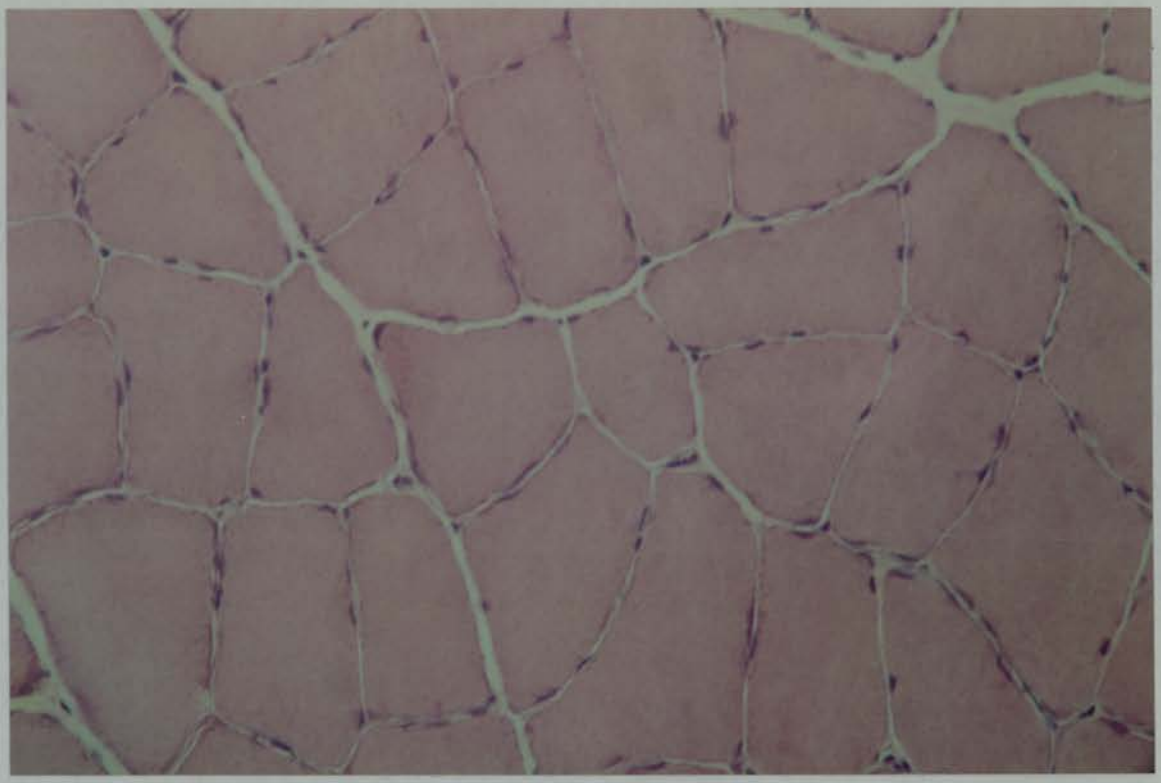

Figure 8.2.1 - Muscle fibre architecture of a normal soleus muscle. Note the size and shape of the muscle fibres and the peripheral location of the nuclei. (H & E, magnification x100).

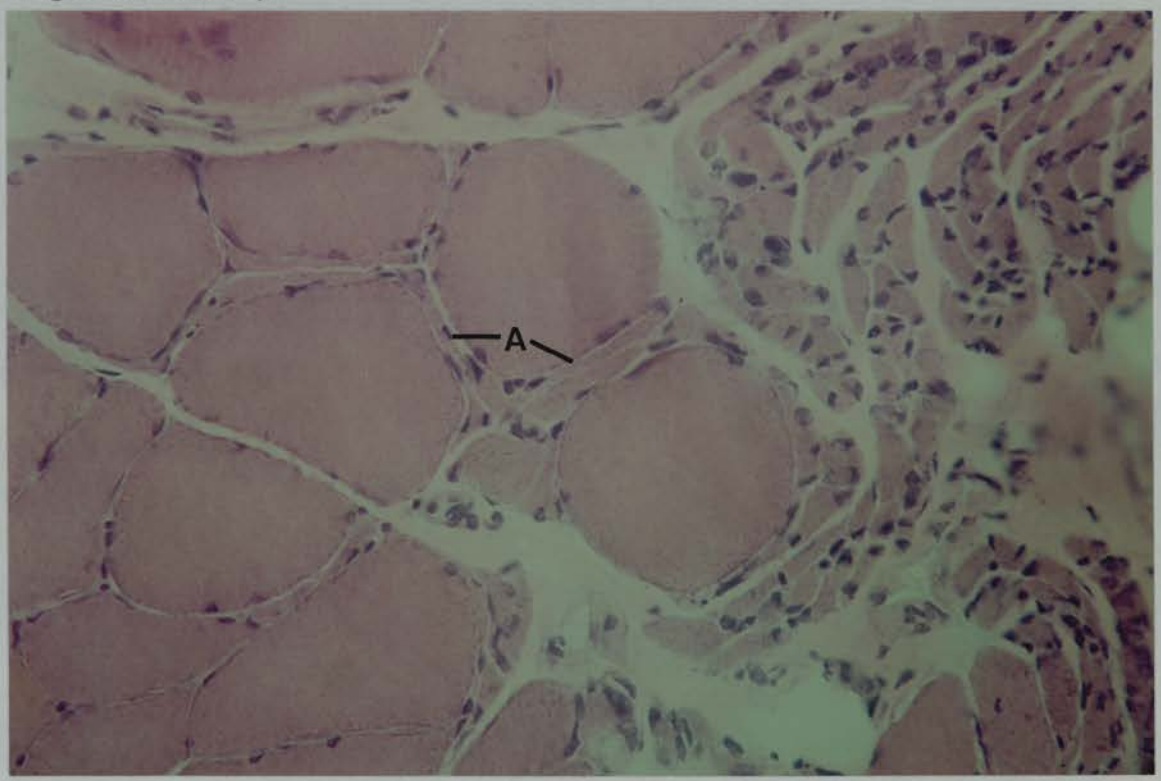

Figure 8.2.2 - Soleus muscle 100 days after transection of the sciatic nerve and repair by a direct epineurial suture. Note the angular fibres (A) between the normal muscle fibres and the large area of atrophied fibres to the right hand side. (H & E, magnification x250).

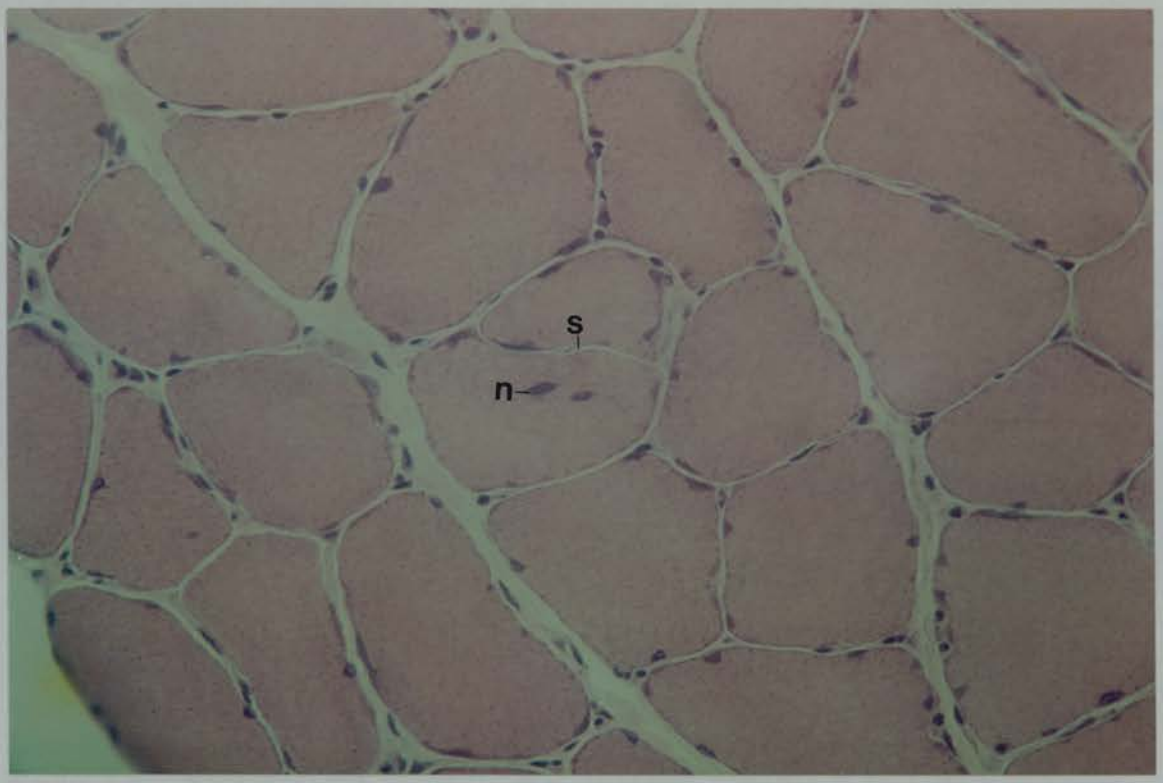

Figure 8.2.3 - Soleus muscle 100 days after transection of the sciatic nerve and repair with a muscle graft. Note the split fibre (s) with internal nuclei (n). (H & E, magnification x250).

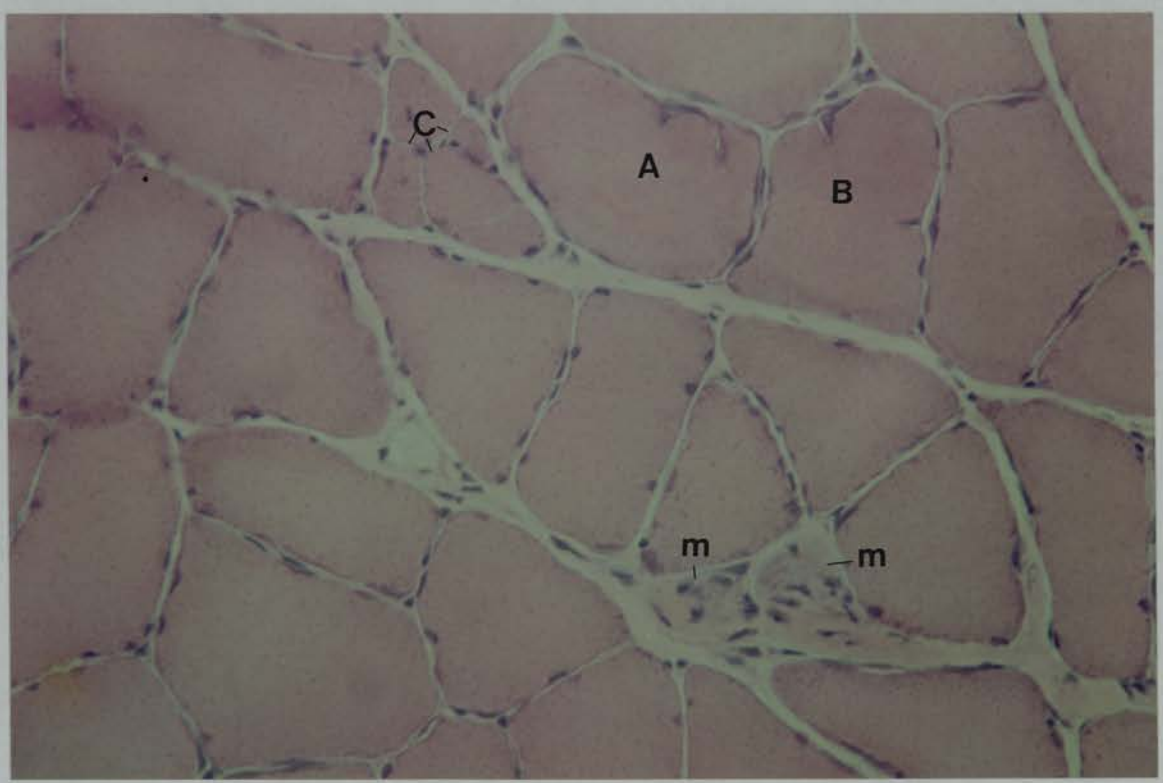

Figure 8.2.4 - Soleus muscle 100 days after transection of the sciatic nerve and repair with a muscle graft. Note fibres (A) and (B) which are in the early stages of splitting, fibre (C) which has fully split several times and the necrotic fibres (m). (H & E, magnification x250).

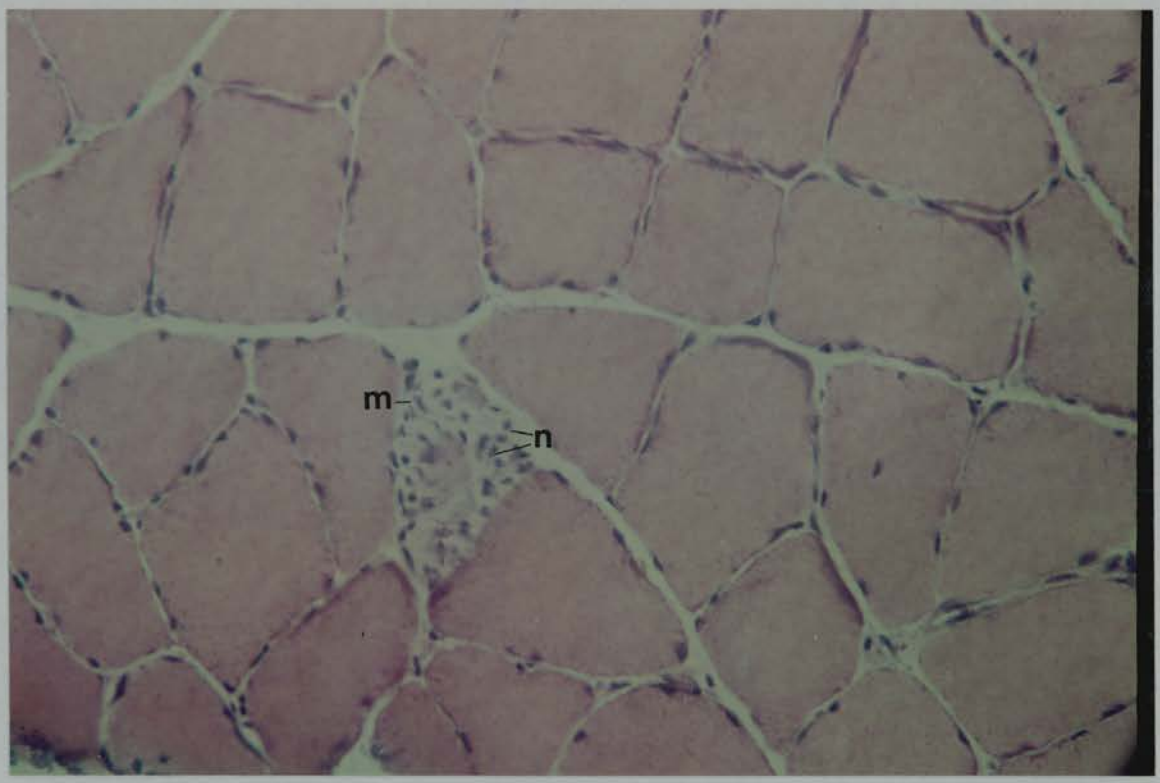

Figure 8.2.5 - Soleus muscle 50 days after transection of the sciatic nerve and repair with a direct epineurial suture. Note the necrotic fibre (m) with the internal nuclei (n). (H & E, magnification x 250).

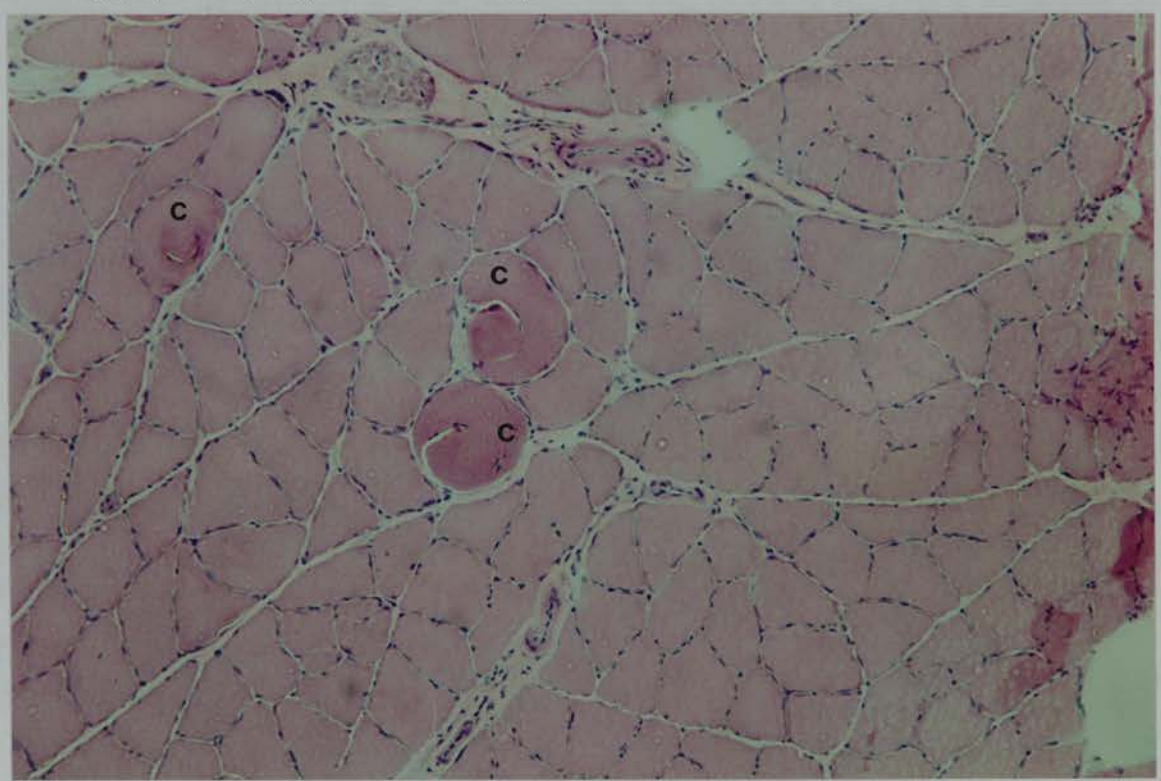

Figure 8.2.6 - Soleus muscle 100 days after transection of the sciatic nerve and repair with a muscle graft. Note the large circular fibres (c). (H & E, magnification x 100)

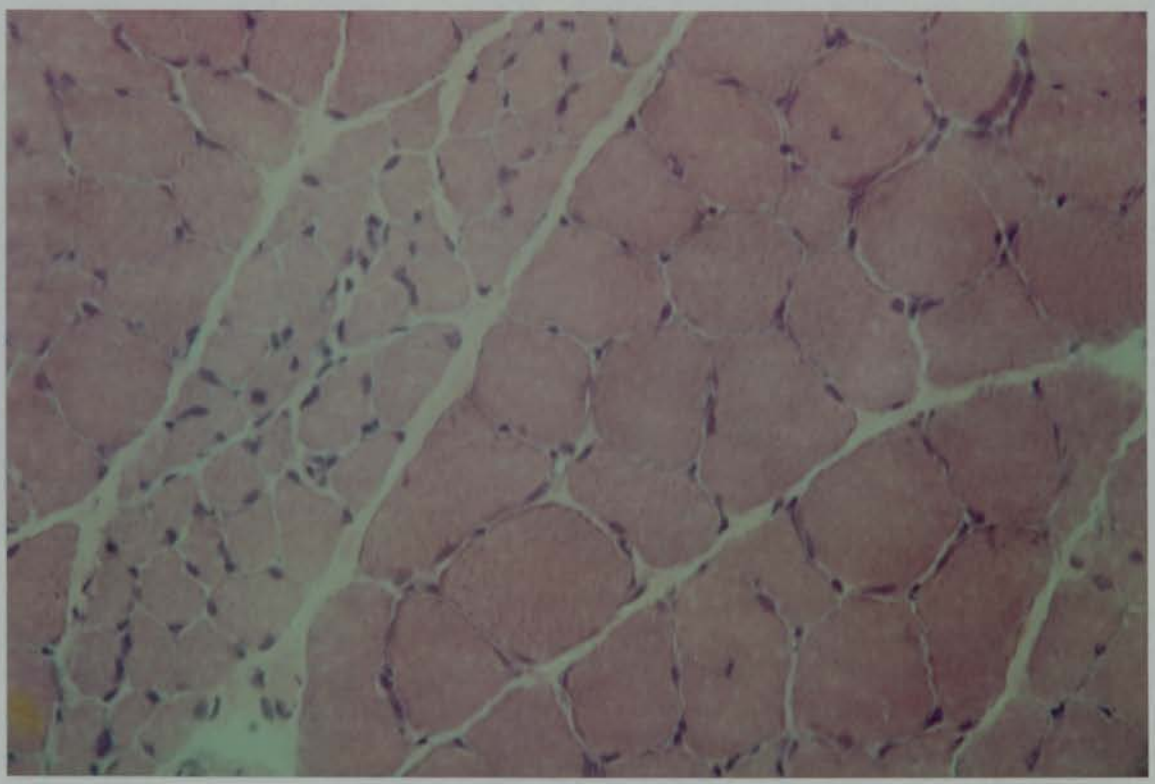

Figure 8.2.7 - EDL 50 days after transection of the sciatic nerve and repair with a direct epineurial suture. Note the fibres in the fascicle to the left of centre, which are markedly atrophied compared to those in the surrounding fascicles. (H & E, magnification x 250).

2. Histochemical fibre types.

(a) Fibre type grouping.

Table 8.2.2 illustrates the presence of fibre type grouping after each type of injury and repair at each of the time periods after operation. After a crush injury to the nerve there was no evidence of fibre type grouping in either the experimental or the contralateral control muscles, at any time after operation. This was in direct contrast to the situation found after transection and repair. Fibre type grouping was evident in all the experimental muscles after transection and repair of the nerve (figures 8.2.10 - 8.2.12), with the exception of the 50 days muscle graft group where the soleus muscle exhibited fibre type grouping but the EDL muscle did not. There was no such fibre type grouping in the contralateral control muscles where the characteristic mosaic pattern of fibre types was maintained - figures 8.2.8 and 8.2.9. The method of repair and the time which had elapsed since operation had no effect on whether fibre type grouping was present or not. Similarly these factors did not affect the degree of fibre type grouping. There was a marked difference in the degree of fibre type grouping between the soleus muscle and the EDL. Experimental EDL muscles consistently averaged 1 or 2 small groups (12-15 fibres) of the same fibre type and a negligible number of large groups (>15 fibres). By contrast, in the soleus, there was an average of 2 to 3 small groups but a much greater number of large groups. Indeed, the degree of fibre type grouping in the soleus was so extensive as to involve groups which covered more than half the fibres on a section. Consequently there was often a change in the fibre type predominance in experimental soleus muscles (see below).

(b) Fibre type predominance.

Table 8.2.3 illustrates the presence of fibre type predominance after each type of injury and repair at each of the time periods after operation. After a nerve crush injury fibre type predominance was the same as in the contralateral control muscles, i.e. type II fibres were predominant in EDL and type I fibres were predominant in the soleus. After transection and repair of the nerve, type II fibres were still predominant in the experimental and contralateral control EDL and type I fibres were still predominant in contralateral control soleus. However the experimental soleus muscle changed from being predominantly type I fibres to predominantly type II. This result was consistent for all repair types and at all time periods after operation with the exception of 50days after the insertion of a nerve graft or muscle graft, 100days after nerve to nerve suture and 200 days after muscle graft. In each of these cases neither fibre type could be said to be predominant. This still indicates an increase in the number of type II fibres present in the experimental soleus muscles since contralateral control muscles contained very few type II fibres. The method of repair and the time elapsed since operation had no effect on the extent of fibre type predominance.

3. Size range of muscle fibres and fascicles.

The size range of the muscle fibres was assessed qualitatively and scored as either 'normal' (fibres within the normal size range) or 'variable' (fibres outwith the normal size range). The normal size range was assessed qualitatively by scanning several contralateral control sections from age and weight matched animals. The results obtained in this way were extremely variable with no consistencies between experimental groups, time periods after operation or even

Days after operation	Repair	Fibre type grouping			
		EDL (E)	EDL (C)	Soleus (E)	Soleus (C)
50 days	Nerve Crush	-	-	-	-
	N-N Suture	*	-	*	-
	Nerve Graft	*	-	*	-
	Muscle Graft	-	-	*	-
100 days	Nerve Crush	-	-	-	-
	N-N Suture	*	-	*	-
	Nerve Graft	*	-	*	-
	Muscle Graft	*	-	*	-
200 days	Nerve Crush	-	-	-	-
	N-N Suture	*	-	*	-
	Nerve Graft	*	-	*	-
	Muscle Graft	*	-	*	-
300 days	Nerve Crush	-	-	-	-
	N-N Suture	*	-	*	-
	Nerve Graft	*	-	*	-
	Muscle Graft	*	-	*	-

Table 8.2.2 - The presence of fibre type grouping, as defined by the enclosed fibre theory of Jennekens et al (1971), in the experimental (E) and contralateral control (C) EDL and soleus after each type of injury and repair and at each time period after operation. (- = absent, * = present).

Days after operation	Repair	Fibre type predominance			
		EDL (E)	EDL (C)	Soleus (E)	Soleus (C)
50 days	Nerve Crush	II	II	I	I
	N-N Suture	II	II	II	I
	Nerve Graft	II	II	-	I
	Muscle Graft	II	II	-	I
100 days	Nerve Crush	II	II	I	I
	N-N Suture	II	II	-	I
	Nerve Graft	II	II	II	I
	Muscle Graft	II	II	II	I
200 days	Nerve Crush	II	II	I	I
	N-N Suture	II	II	II	I
	Nerve Graft	II	II	II	I
	Muscle Graft	II	II	-	I
300 days	Nerve Crush	II	II	I	I
	N-N Suture	II	II	II	I
	Nerve Graft	II	II	II	I
	Muscle Graft	II	II	II	I

Table 8.2.3 - Fibre type predominance in the experimental (E) and contralateral control (C) EDL and soleus after each type of injury and repair and at each time period after operation. (I = type I fibres are predominant, II = type II fibres are predominant, - = approximately equal numbers of type I and type II fibres)

Figure 8.2.8 - Normal soleus muscle - note the predominance of light type I fibres and the random distribution of the dark type II fibres. (ATPase pH 10.2, magnification x 100).

Figure 8.2.9 - Normal EDL muscle - note the predominance of dark type II fibres and the random distribution of the light type I fibres. (ATPase pH 10.2, magnification x 100).

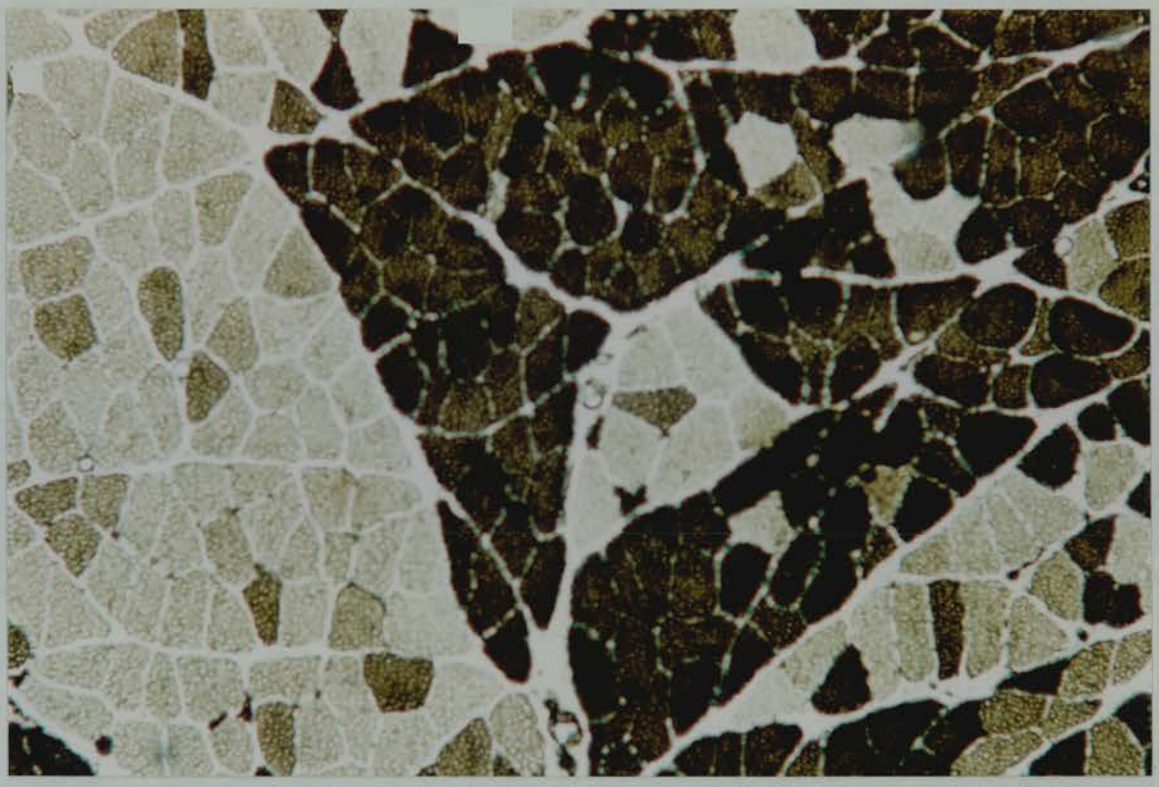

Figure 8.2.10 - Soleus muscle 50 days after the transection of the sciatic nerve and repair with a nerve graft. Note the large groups of type II fibres. (ATPase pH 10.2, magnification x 100).

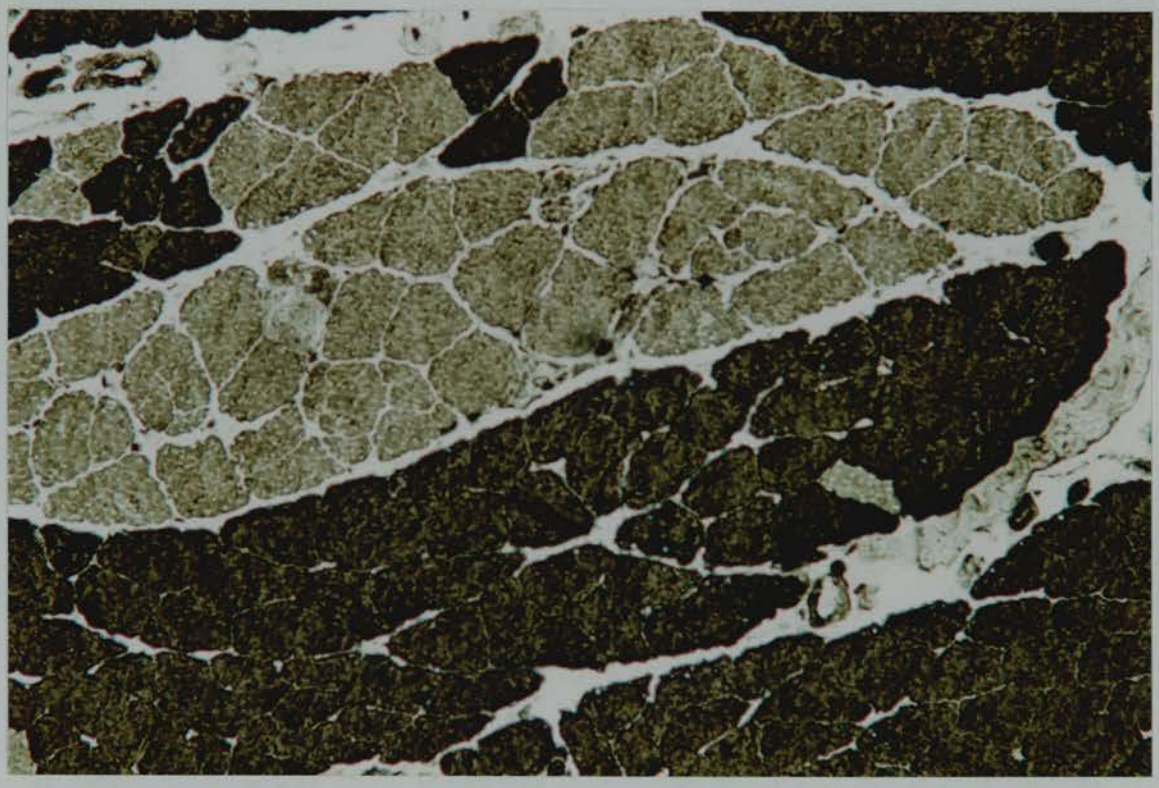

Figure 8.2.11 - EDL 300 days after the transection of the sciatic nerve and repair with a nerve graft. Note the large group of type I fibres. (ATPase pH 10.2, magnification x 100).

Figure 8.2.12 - Soleus muscle 100 days after the transection of the sciatic nerve and repair with a direct epineurial suture. Note the fascicle on the LHS which is made up entirely of type II fibres, the fascicle in the centre which is made up entirely of type I fibres and the fascicles on the RHS which have the normal mosaic pattern of type I and type II fibres. (ATPase pH 10.2, magnification x 100).

between contralateral control muscles. It was decided that it would be much more accurate to measure the size of the muscle fibres quantitatively.

8.3 Quantitative assessment of muscles.

1. Measurement of muscle fibres.

(a) Minimum diameter of muscle fibres.

The mean minimum fibre diameter of the experimental muscles was generally less than or equal to the contralateral control values at all time periods after operation. This is illustrated in table 8.3.1. and on figure 8.3.1 (A - P). On considering the mean minimum fibre diameter of type I fibres in EDL, the difference between experimental and contralateral control values failed to reach statistical significance after injury and repair - see table 8.3.2. This was true for all repair methods and time periods with the exception of 50 and 200 days after a nerve crush injury ($p<0.05$ in each case) and 200 days after the insertion of a muscle graft ($p<0.01$). In the soleus muscle there was no significant difference between the experimental and contralateral control mean minimum fibre diameter measurements at any time after a nerve crush injury. The difference was significant at the 1% level 50 days after repair with a nerve graft and 100 days after a nerve to nerve suture but at no other time period. After repair by means of a muscle graft the difference in mean minimum diameter of type I fibres between experimental and contralateral control soleus muscles was significantly different at 50 days ($p<0.01$), 100 days ($p<0.05$) and 300 days ($p<0.01$) after operation. On considering the mean minimum fibre diameter of type II fibres in the EDL, the difference between experimental and contralateral control values reached statistical significance 50

days after the insertion of a muscle graft ($p<0.01$), 100 days after the insertion of a nerve graft ($p<0.05$), 200 days after a nerve crush injury ($p<0.05$) and 50 and 100 days after repair by means of a direct epineurial suture ($p<0.01$ and $p<0.001$ respectively). The soleus muscle showed a significant decrease in the mean minimum diameter of type II fibres in experimental muscles compared to contralateral controls at several time periods after each of the different methods of injury and repair. This difference was significant at the 5% level 200 days after the insertion of a muscle graft and 50 and 300 days after a nerve crush injury, at the 1% level 50 days after the insertion of a nerve graft and 300 days after a direct epineurial suture and at the 0.1% level 50 and 100 days after the insertion of a muscle graft, 300 days after the insertion of a nerve graft and 100 days after a direct epineurial suture - see table 8.3.2.

When considering the mean minimum fibre diameter of type I fibres in the experimental EDL, with respect to repair, there was little difference between the values. Indeed the difference in the mean minimum type I fibre diameter failed to reach statistical significance on comparing each of the repair methods with one another at 50, 100 and 300 days after operation - see table 8.3.3. At 200 days after operation the mean minimum diameter of type I fibres seen after a nerve crush injury or the insertion of a muscle graft was significantly less than after the insertion of a nerve graft or repair by direct epineurial suture ($p<0.05$ in each case). When considering the mean minimum fibre diameter of type II fibres in experimental EDL after each of the methods of injury and repair there was no statistical significance 200 and 300 days after operation. At 100 days after operation the minimum diameter of type II fibres was significantly less after repair

			Minimum Diameter (μm)							
			Experimental muscle				Contralateral control muscle			
			Fibre type I		Fibre type II		Fibre type I		Fibre type II	
Duratio	Repair	Muscle	Mean	S.D	Mean	S.D	Mean	S.D	Mean	S.D
50 days	MG	EDL	31.58	7.92	26.80	2.83	34.42	3.56	42.44	3.90
	NC		35.79	2.98	35.75	5.15	37.53	3.91	37.36	4.34
	NG		35.99	1.73	37.59	1.76	34.34	2.86	40.04	3.09
	NN		39.85	8.65	37.95	4.64	44.67	12.87	52.07	6.18
	MG	Soleus	31.37	8.45	28.19	6.55	56.59	5.74	46.81	7.03
	NC		51.05	6.58	43.33	6.29	56.65	1.43	56.81	5.62
	NG		41.69	6.41	36.25	5.85	55.62	2.36	52.27	5.12
	NN		46.09	5.49	44.29	5.43	44.98	8.19	49.31	4.84
100 days	MG	EDL	35.28	7.53	37.65	8.29	35.88	2.93	45.80	7.12
	NC		33.77	3.64	43.57	2.01	35.71	3.22	45.82	4.29
	NG		38.62	13.25	45.32	3.99	35.66	6.65	48.71	5.08
	NN		33.20	2.98	38.93	2.96	35.79	3.30	47.09	4.03
	MG	Soleus	47.67	6.92	40.00	2.32	54.84	3.87	53.75	4.08
	NC		54.77	1.51	54.64	5.18	51.60	5.40	59.56	6.18
	NG		50.73	3.81	47.11	7.01	51.51	8.51	52.21	8.04
	NN		47.88	6.96	40.64	2.40	57.76	2.65	57.76	2.32
200 days	MG	EDL	31.72	2.93	38.70	4.43	34.60	3.64	42.75	4.00
	NC		32.07	1.69	44.15	2.17	35.45	2.32	47.92	4.20
	NG		35.43	1.72	41.34	3.62	34.92	2.61	42.19	5.41
	NN		36.19	2.34	43.64	2.65	35.03	2.81	46.54	2.88
	MG	Soleus	49.54	10.92	44.59	4.84	54.15	2.02	39.25	8.08
	NC		53.66	2.88	45.20	5.54	53.51	2.05	42.76	8.50
	NG		53.14	9.56	39.86	3.40	50.28	8.77	48.29	14.51
	NN		52.10	6.45	50.09	4.42	56.87	4.32	49.17	5.01
300 days	MG	EDL	31.31	3.67	47.61	7.12	34.93	5.57	51.52	3.85
	NC		27.24	4.65	26.80	2.83	44.32	2.47	43.13	3.22
	NG		37.17	10.43	46.24	5.76	33.30	2.19	47.62	5.00
	NN		30.87	7.74	41.70	2.52	35.04	3.21	42.97	4.28
	MG	Soleus	47.46	8.32	47.22	4.44	56.87	2.34	51.97	11.53
	NC		56.30	9.02	53.25	10.94	53.24	4.56	60.26	9.33
	NG		48.98	19.19	46.91	5.01	55.23	6.36	61.10	4.57
	NN		53.64	6.64	43.25	2.62	53.30	6.84	53.15	5.32

Table 8.3.1 - The mean minimum fibre diameter of type I and type II muscle fibres of the experimental and contralateral control EDL and soleus, after each type of injury and repair at each time period after operation. (MG = muscle graft, NC = nerve crush, NG = nerve graft, NN = nerve to nerve suture).

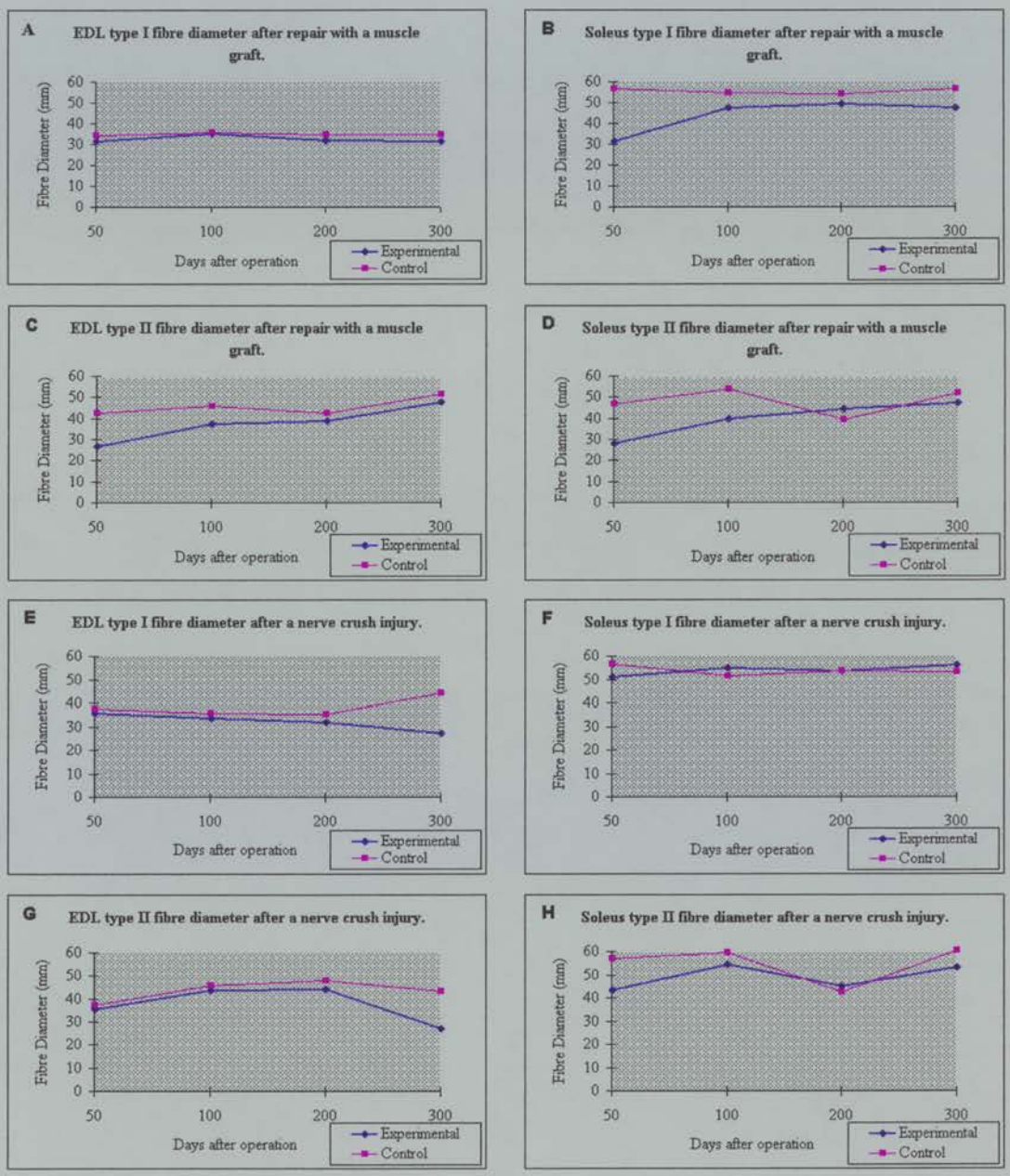

Figure 8.3.1 (A - H) - The changes in the mean minimum fibre diameter of type I and type II muscle fibres of the experimental and contralateral control muscles at each time period after operation. The changes after repair of the sciatic nerve with a muscle graft are shown on graphs (A) to (D) and after a nerve crush injury on graphs (E) to (H).

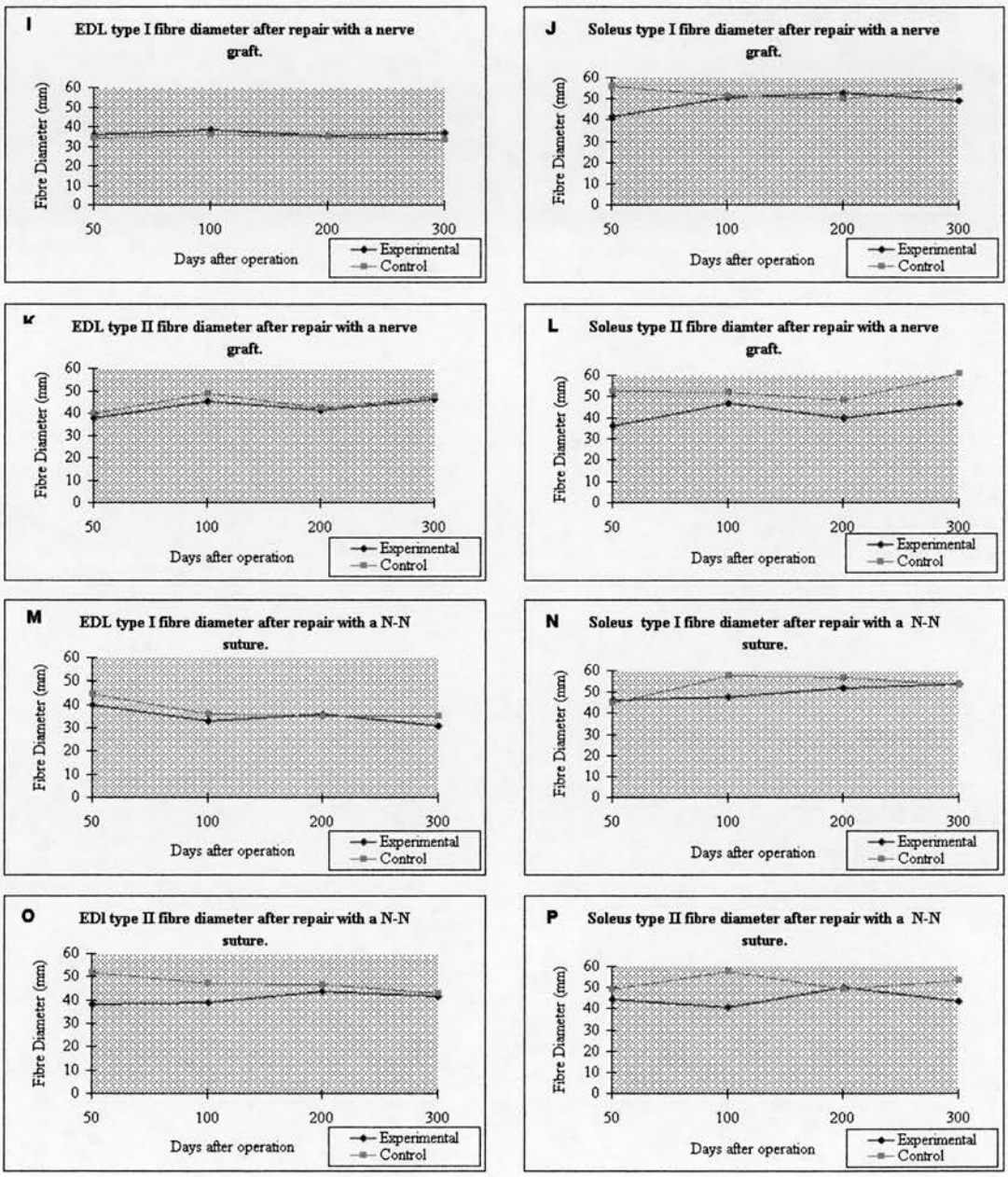

Figure 8.3.1 (I - P) - The changes in the mean minimum fibre diameter of type I and type II muscle fibres of the experimental and contralateral control muscles at each time period after operation. The changes after repair of the sciatic nerve with a nerve graft are shown on graphs (I) to (L) and after repair with a direct epineurial suture on graphs (M) to (P).

(A)

Repair	Muscle	Days after operation			
		50	100	200	300
Muscle Graft	EDL	N. S.	N.S.	$p<0.01$	N.S.
	Soleus	$p<0.01$	$p<0.05$	N.S.	$p<0.05$
Nerve Crush	EDL	$p<0.05$	N.S.	$p<0.05$	N.S.
	Soleus	N.S.	N.S.	N.S.	N.S.
Nerve Graft	EDL	N.S.	N.S.	N.S.	N.S.
	Soleus	$p<0.01$	N.S.	N.S.	N.S.
N-N Suture	EDL	N.S.	N.S.	N.S.	N.S.
	Soleus	N.S.	$p<0.01$	N.S.	N.S.

(B)

Repair	Muscle	Days after operation			
		50	100	200	300
Muscle Graft	EDL	$p<0.01$	N.S.	N.S.	N.S.
	Soleus	$p<0.001$	$p<0.001$	$p<0.05$	N.S.
Nerve Crush	EDL	N.S.	N.S.	$p<0.05$	N.S.
	Soleus	$p<0.05$	N.S.	N.S.	$p<0.05$
Nerve Graft	EDL	N.S.	$p<0.05$	N.S.	N.S.
	Soleus	$p<0.01$	N.S.	N.S.	$p<0.001$
N-N Suture	EDL	$p<0.01$	$p<0.001$	N.S.	N.S.
	Soleus	N.S.	$p<0.001$	N.S.	$p<0.01$

Table 8.3.2 - **(A)** The significance values for the comparison of the mean minimum diameter of **type I** muscle fibres in the experimental and contralateral control muscles after each type of injury and repair and at each time period after operation. (N.S. = no significant difference).

(B) The significance values for the comparison of the mean minimum diameter of **type II** muscle fibres in the experimental and contralateral control muscles after each type of injury and repair and at each time period after operation. (N.S. = no significant difference).

(A)

Repair	50 Days - Type I Fibres			
	Muscle Graft	Nerve Crush	Nerve Graft	N-N Suture
Muscle Graft	*	*	*	*
Nerve Crush	N.S.	*	*	*
Nerve Graft	N.S.	N.S.	*	*
N-N Suture	N.S.	N.S.	N.S.	*

(B)

Repair	100 Days - Type I Fibres			
	Muscle Graft	Nerve Crush	Nerve Graft	N-N Suture
Muscle Graft	*	*	*	*
Nerve Crush	N.S.	*	*	*
Nerve Graft	N.S.	N.S.	*	*
N-N Suture	N.S.	N.S.	N.S.	*

(C)

Repair	200 Days - Type I Fibres			
	Muscle Graft	Nerve Crush	Nerve Graft	N-N Suture
Muscle Graft	*	*	*	*
Nerve Crush	N.S.	*	*	*
Nerve Graft	$p<0.05$	$p<0.01$	*	*
N-N Suture	$p<0.05$	$p<0.05$	N.S.	*

(D)

Repair	300 Days - Type I Fibres			
	Muscle Graft	Nerve Crush	Nerve Graft	N-N Suture
Muscle Graft	*	*	*	*
Nerve Crush	N.S.	*	*	*
Nerve Graft	N.S.	N.S.	*	*
N-N Suture	N.S.	N.S.	N.S.	*

Table 8.3.3 - The significance values for the comparison of the mean minimum diameter of **type I** muscle fibres in the experimental **EDL** after each type of injury and repair. (A) 50 days, (B) 100 days, (C) 200 days and (D) 300 days after operation. (N.S. = no significant difference).

(E)

Repair	50 Days - Type II Fibres			
	Muscle Graft	Nerve Crush	Nerve Graft	N-N Suture
Muscle Graft	*	*	*	*
Nerve Crush	$p<0.01$	*	*	*
Nerve Graft	$p<0.01$	N.S.	*	*
N-N Suture	$p<0.001$	N.S.	N.S.	*

(F)

Repair	100 Days - Type II Fibres			
	Muscle Graft	Nerve Crush	Nerve Graft	N-N Suture
Muscle Graft	*	*	*	*
Nerve Crush	N.S.	*	*	*
Nerve Graft	N.S.	N.S.	*	*
N-N Suture	N.S.	$p<0.01$	$p<0.01$	*

(G)

Repair	200 Days - Type II Fibres			
	Muscle Graft	Nerve Crush	Nerve Graft	N-N Suture
Muscle Graft	*	*	*	*
Nerve Crush	N.S.	*	*	*
Nerve Graft	N.S.	N.S.	*	*
N-N Suture	N.S.	N.S.	N.S.	*

(H)

Repair	300 Days - Type II Fibres			
	Muscle Graft	Nerve Crush	Nerve Graft	N-N Suture
Muscle Graft	*	*	*	*
Nerve Crush	N.S.	*	*	*
Nerve Graft	N.S.	N.S.	*	*
N-N Suture	N.S.	N.S.	N.S.	*

Table 8.3.3 (continued) - The significance values for the comparison of the mean minimum diameter of **type II** muscle fibres in the experimental **EDL** after each type of injury and repair. (E) 50 days, (F) 100 days, (G) 200 days and (H) 300 days after operation. (N.S. = no significant difference).

with a direct epineurial suture than after a nerve crush injury or the insertion of a nerve graft. This difference was significant at the 1% level in each case. At 50 days after operation repair by the insertion of a muscle graft resulted in a significantly smaller mean minimum diameter of type II fibres than after any other method of injury and repair. This difference was significant at the 1% level on comparing the muscle graft group with the nerve crush and nerve graft group and at the 0.1% level on comparison with the direct epineurial suture group.

In the soleus muscle the mean minimum diameter of type I fibres was not significantly different after each of the different methods of injury and repair at 100, 200 and 300 days after operation - see table 8.3.4. At 50 days after operation repair by means of a muscle graft resulted in a significantly smaller mean diameter than after any other type of injury and repair ($p<0.01$ in each case). The nerve crush injury also resulted in a significantly greater minimum diameter than after repair by means of the insertion of a nerve graft ($p<0.05$). The minimum diameter of type II fibres in the experimental soleus muscle was significantly smaller after repair by the insertion of a muscle graft than after any other method of injury and repair ($p<0.01$ in each case). At 100 and 200 days after operation the mean minimum fibre diameter was significantly different after repair by means of a nerve graft compared to repair with a muscle graft or a direct epineurial suture ($p<0.05$ in each case). However at 100 days after operation, repair by a nerve graft resulted in a significantly greater fibre diameter than the other methods of repair whereas at 200 days after operation the converse was true.

Figure 8.3.1 (graphs A - P) clearly shows there was generally little difference

in the mean minimum diameter of fibres between 50 and 300 days after operation, although in some cases a slight increases in fibre diameter does occur. The minimum diameter of type I fibres in the experimental EDL showed no significant difference between 50 and 300 days after operation in any of the groups which involved the transection of the nerve. The difference between the minimum diameter of type I fibres at 50 and 300 days after a nerve crush injury were statistically significant at the 1% level. Type II fibres in the experimental EDL all showed a significant increase in minimum fibre diameter from 50 to 300 days after operation. This difference was significant at the 1% level after repair by means of a muscle graft and at the 5% level after a nerve crush injury and after repair by a direct epineurial suture or a nerve graft. In the soleus muscle there was a significant increase in minimum fibre diameter of type I fibres from 50 to 300 days after transection of the nerve and repair by means of a muscle graft or direct epineurial suture ($p<0.05$ in each case). There was no such significant increase with respect to time after a nerve crush injury or after repair by a nerve graft. There was a significant increase in minimum fibre diameter of type II fibres from 50 to 300 days after transection of the nerve and repair by means of a graft. This difference was significant at the 1% level after repair with a muscle graft and at the 5% level after repair with a nerve graft. There was no such significant increase with respect to time after a nerve crush injury or after repair by means of a direct epineurial suture.

In general, in the EDL, the mean minimum diameter of type II fibres tended to be greater than for type I fibres, this was true both for the experimental and

(A)

Repair	50 Days - Type I Fibres			
	Muscle Graft	Nerve Crush	Nerve Graft	N-N Suture
Muscle Graft	*	*	*	*
Nerve Crush	$p<0.01$	*	*	*
Nerve Graft	$p<0.01$	$p<0.05$	*	*
N-N Suture	$p<0.01$	N.S.	N.S.	*

(B)

Repair	100 Days - Type I Fibres			
	Muscle Graft	Nerve Crush	Nerve Graft	N-N Suture
Muscle Graft	*	*	*	*
Nerve Crush	N.S.	*	*	*
Nerve Graft	N.S.	N.S.	*	*
N-N Suture	N.S.	N.S.	N.S.	*

(C)

Repair	200 Days - Type I Fibres			
	Muscle Graft	Nerve Crush	Nerve Graft	N-N Suture
Muscle Graft	*	*	*	*
Nerve Crush	N.S.	*	*	*
Nerve Graft	N.S.	N.S.	*	*
N-N Suture	N.S.	N.S.	N.S.	*

(D)

Repair	300 Days - Type I Fibres			
	Muscle Graft	Nerve Crush	Nerve Graft	N-N Suture
Muscle Graft	*	*	*	*
Nerve Crush	N.S.	*	*	*
Nerve Graft	N.S.	N.S.	*	*
N-N Suture	N.S.	N.S.	N.S.	*

Table 8.3.4 - The significance values for the comparison of the mean minimum diameter of **type I** muscle fibres in the experimental **soleus** after each type of injury and repair.(A) 50 days, (B) 100 days, (C) 200 days and (D) 300 days after operation. (N.S. = no significant difference).

(E)

Repair	50 Days - Type II Fibres			
	Muscle Graft	Nerve Crush	Nerve Graft	N-N Suture
Muscle Graft	*	*	*	*
Nerve Crush	$p<0.01$	*	*	*
Nerve Graft	$p<0.01$	N.S.	*	*
N-N Suture	$p<0.01$	N.S.	$p<0.05$	*

(F)

Repair	100 Days - Type II Fibres			
	Muscle Graft	Nerve Crush	Nerve Graft	N-N Suture
Muscle Graft	*	*	*	*
Nerve Crush	N.S.	*	*	*
Nerve Graft	$p<0.05$	$p<0.05$	*	*
N-N Suture	N.S.	$p<0.001$	$p<0.05$	*

(G)

Repair	200 Days - Type II Fibres			
	Muscle Graft	Nerve Crush	Nerve Graft	N-N Suture
Muscle Graft	*	*	*	*
Nerve Crush	N.S.	*	*	*
Nerve Graft	$p<0.05$	N.S.	*	*
N-N Suture	N.S.	N.S.	$p<0.01$	*

(H)

Repair	300 Days - Type II Fibres			
	Muscle Graft	Nerve Crush	Nerve Graft	N-N Suture
Muscle Graft	*	*	*	*
Nerve Crush	N.S.	*	*	*
Nerve Graft	N.S.	N.S.	*	*
N-N Suture	N.S.	N.S.	N.S.	*

Table 8.3.4 (continued) - The significance values for the comparison of the mean minimum diameter of **type II** muscle fibres in the experimental **soleus** after each type of injury and repair. (E) 50 days, (F) 100 days, (G) 200 days and (H) 300 days after operation. (N.S. = no significant difference).

contralateral control muscles. This difference was statistically significant in both the experimental and contralateral control muscles 100, 200 and 300 days after a nerve crush injury and after repair by a direct epineurial suture - see table 8.3.5. After repair by means of the insertion of a graft this difference was statistically significant at all time. periods after operation in the contralateral control EDL. In the experimental muscle this difference was only significant 200 days after the insertion of a nerve graft ($p<0.05$), and at 200 and 300 days after the insertion of a muscle graft ($p<0.05$ and $p<0.01$ respectively). The mean minimum diameter of type I fibres tended to be greater than type II fibres in the soleus, this was true both for the experimental and contralateral control muscles. This difference reached statistical significance in both the experimental and contralateral control soleus after several of the methods of injury and repair and at several of the time periods after operation. There was no consistency between the groups which failed to reach statistical significance in terms of the difference in minimum fibre diameter between type I and type II fibres in the experimental and contralateral control muscles.

With the exception of 2 out of the 32 experimental groups, the standard deviation of the mean minimum fibre diameter was less than 0.25 of the value of the mean diameter in the contralateral control muscles. This indicates that there was no abnormal variability in muscle fibre size in the contralateral control muscles (Dubowitz 1985). Similarly in the experimental muscles, the standard deviation of the mean minimum fibre diameter was less than 0.25 of the value of the mean diameter in all but 5 of the 32 experimental groups. This indicates that there was

negligible abnormal variability in muscle fibre size in the experimental muscles. When this is considered together with the fact that the mean minimum fibre diameter was less in the experimental muscles it may be concluded that all muscle fibres had undergone a similar degree of atrophy during the denervation/reinnervation process.

(b) Form Factor

The form factors of type I and type II fibres were assessed independently. On analysis of the results there was little or no difference in the form factor values for the two fibre types ($p > 0.05$ in each case) and hence for all subsequent analyses the mean of the two values was used. The results are shown in table 8.3.6 and in figure 8.3.2 (A -H).

There was no significant difference between the form factors of muscle fibres when comparing experimental and contralateral control muscles. When considering the form factor of the muscle fibres after each of the methods of injury and repair, the vast majority of the groups showed no significant difference. In the very few cases where there was a significant difference between the repair methods there was no consistency between groups - see table 8.3.7. For example the form factor in the nerve crush group was significantly greater than for the nerve to nerve suture group in the soleus 100 days after operation ($p < 0.05$), by 200 days the nerve to nerve suture group was significantly greater than the nerve crush group ($p < 0.05$) and by 300 days there was no significant difference between the two groups. Similarly when considering the form factor of the muscle fibres at each of the time periods after operation the vast majority of the groups showed no significant

(A)

		Days after Operation			
Repair	**Muscle**	**50**	**100**	**200**	**300**
Muscle Graft	EDL	N.S.	N.S.	$p<0.05$	$p<0.01$
	Soleus	N.S.	$p<0.05$	N.S.	N.S.
Nerve Crush	EDL	N.S.	$p<0.001$	$p<0.001$	$p<0.01$
	Soleus	$p<0.01$	N.S.	$p<0.05$	N.S.
Nerve Graft	EDL	N.S.	N.S.	$p<0.05$	N.S.
	Soleus	$p<0.01$	N.S.	$p<0.05$	N.S.
N-N Suture	EDL	N.S.	$p<0.01$	$p<0.001$	$p<0.01$
	Soleus	N.S.	$p<0.05$	N.S.	$p<0.05$

(B)

		Days after Operation			
Repair	**Muscle**	**50**	**100**	**200**	**300**
Muscle Graft	EDL	$p<0.05$	$p<0.01$	$p<0.001$	$p<0.001$
	Soleus	$p<0.05$	N.S.	$p<0.01$	N.S.
Nerve Crush	EDL	N.S.	$p<0.001$	$p<0.001$	$p<0.001$
	Soleus	N.S.	$p<0.05$	$p<0.05$	N.S.
Nerve Graft	EDL	$p<0.05$	$p<0.001$	$p<0.01$	$p<0.01$
	Soleus	N.S.	N.S.	N.S.	$p<0.05$
N-N Suture	EDL	N.S.	$p<0.001$	$p<0.01$	$p<0.01$
	Soleus	N.S.	N.S.	$p<0.05$	N.S.

Table 8.3.5 - (A) The significance values for the comparison of the mean minimum diameter of type I and type II fibres in the **experimental** muscles, after each type of injury and repair and at each time period after operation. (N.S. = no significant difference).

(B) The significance values for the comparison of the mean minimum diameter of type I and type II fibres in the **contralateral control** muscle, after each type of injury and repair and at each time period after operation. (N.S. = no significant difference).

			Form Factor							
			Experimental muscle				Contralateral control muscle			
			Fibre type I		Fibre type II		Fibre type I		Fibre type II	
Duratio	Repair	Muscle	Mean	S.D	Mean	S.D	Mean	S.D	Mean	S.D
50 days	MG	EDL	0.81	0.03	0.76	0.02	0.75	0.05	0.78	0.10
	NC		0.77	0.01	0.79	0.05	0.78	0.09	0.75	0.03
	NG		0.79	0.03	0.78	0.01	0.77	0.03	0.75	0.03
	NN		0.78	0.07	0.75	0.03	0.73	0.05	0.75	0.09
	MG	Soleus	0.80	0.11	0.70	0.10	0.71	0.02	0.76	0.07
	NC		0.72	0.02	0.72	0.02	0.71	0.01	0.72	0.01
	NG		0.73	0.03	0.73	0.04	0.71	0.01	0.71	0.02
	NN		0.73	0.04	0.75	0.05	0.77	0.09	0.72	0.03
100 days	MG	EDL	0.79	0.05	0.76	0.03	0.78	0.02	0.75	0.01
	NC		0.76	0.06	0.76	0.02	0.75	0.04	0.76	0.03
	NG		0.72	0.03	0.71	0.03	0.72	0.08	0.78	0.06
	NN		0.74	0.04	0.72	0.03	0.73	0.08	0.76	0.04
	MG	Soleus	0.71	0.02	0.72	0.01	0.72	0.01	0.70	0.02
	NC		0.78	0.12	0.73	0.01	0.75	0.08	0.71	0.01
	NG		0.71	0.03	0.69	0.02	0.69	0.06	0.70	0.03
	NN		0.69	0.01	0.67	0.04	0.71	0.05	0.67	0.01
200 days	MG	EDL	0.74	0.04	0.74	0.02	0.78	0.06	0.75	0.02
	NC		0.73	0.06	0.74	0.03	0.74	0.03	0.74	0.01
	NG		0.75	0.03	0.76	0.01	0.77	0.03	0.80	0.08
	NN		0.75	0.02	0.74	0.14	0.77	0.05	0.74	0.03
	MG	Soleus	0.74	0.05	0.73	0.02	0.70	0.02	0.67	0.10
	NC		0.71	0.02	0.67	0.04	0.72	0.04	0.72	0.08
	NG		0.78	0.10	0.74	0.03	0.71	0.03	0.66	0.13
	NN		0.73	0.03	0.73	0.02	0.73	0.03	0.70	0.11
300 days	MG	EDL	0.69	0.06	0.78	0.12	0.72	0.06	0.77	0.11
	NC		0.59	0.12	0.76	0.02	0.74	0.05	0.79	0.09
	NG		0.77	0.13	0.75	0.02	0.75	0.03	0.77	0.12
	NN		0.67	0.14	0.75	0.03	0.76	0.05	0.75	0.01
	MG	Soleus	0.71	0.02	0.78	0.12	0.74	0.06	0.64	0.07
	NC		0.74	0.04	0.74	0.03	0.77	0.09	0.70	0.09
	NG		0.75	0.04	0.72	0.01	0.72	0.01	0.72	0.05
	NN		0.72	0.04	0.72	0.03	0.72	0.03	0.73	0.07

Table 8.3.6 - The form factor of muscle fibres of the experimental and contralateral control EDL and soleus, after each type of injury and repair and at each time period after operation. (MG = muscle graft, NC = nerve crush, NG = nerve graft, NN = nerve to nerve suture).

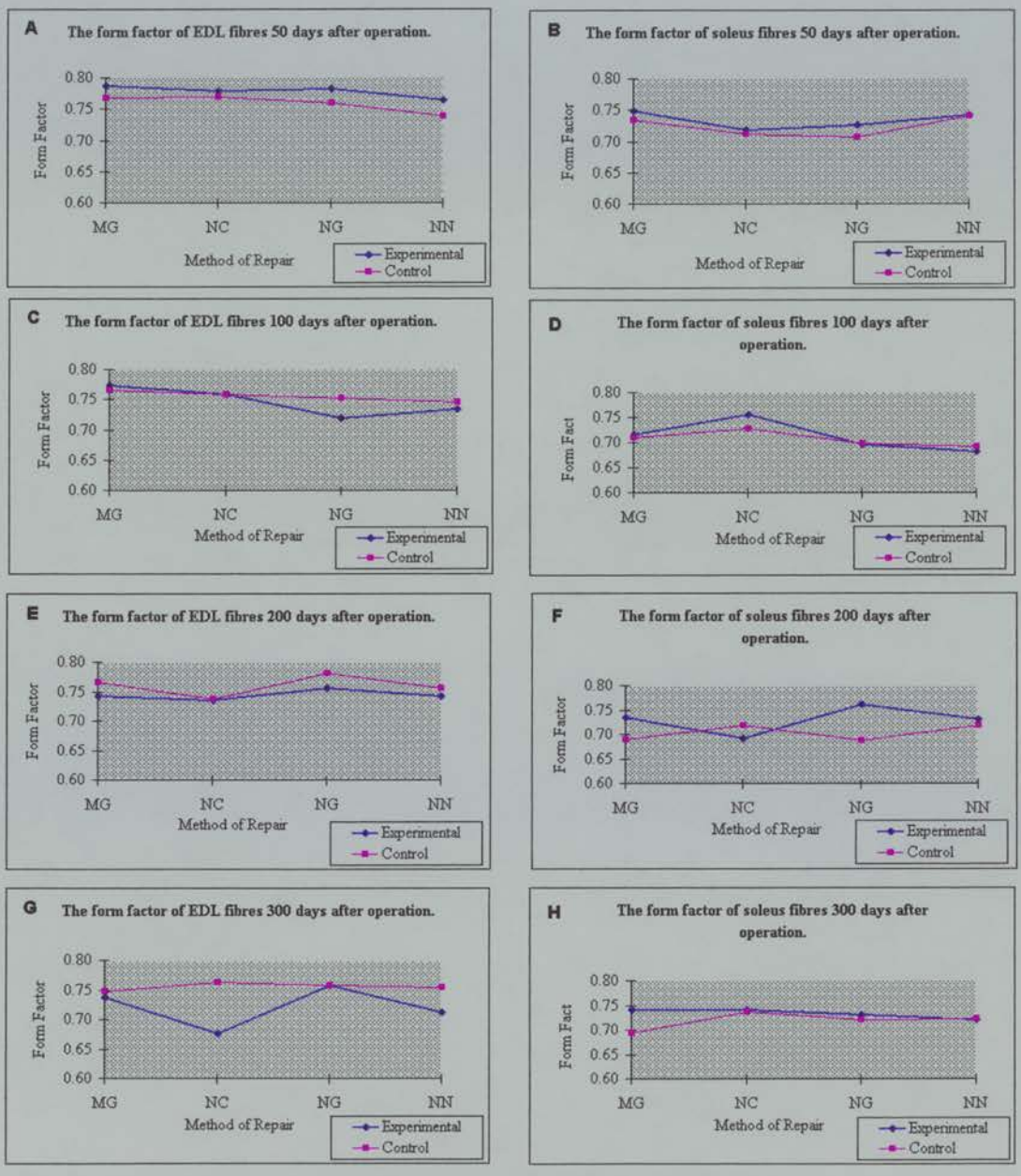

Figure 8.3.2 (A - H) - The changes in the form factor of muscle fibres of the experimental and contralateral control muscles, after each type of injury and repair and at each time period after operation. The changes in the form factor of EDL and soleus muscle fibres 50 days after operation are shown in graphs (A) and (B), 100 days after operation in graphs (C) and (D), 200 days after operation in graphs (E) and (F) and 300 days after operation in graphs (G) and (H). (MG = muscle graft, NC = nerve crush, NG = nerve graft, NN = direct epineurial suture).

(A)

Repair	50 Days			
	Muscle Graft	Nerve Crush	Nerve Graft	N-N Suture
Muscle Graft	*	*	*	*
Nerve Crush	N.S.	*	*	*
Nerve Graft	N.S.	N.S.	*	*
N-N Suture	N.S.	N.S.	N.S.	*

(B)

Repair	100 Days			
	Muscle Graft	Nerve Crush	Nerve Graft	N-N Suture
Muscle Graft	*	*	*	*
Nerve Crush	N.S.	*	*	*
Nerve Graft	N.S.	N.S.	*	*
N-N Suture	$p< 0.05$	$p< 0.05$	N.S.	*

(C)

Repair	200 Days			
	Muscle Graft	Nerve Crush	Nerve Graft	N-N Suture
Muscle Graft	*	*	*	*
Nerve Crush	N.S.	*	*	*
Nerve Graft	N.S.	N.S.	*	*
N-N Suture	N.S.	N.S.	N.S.	*

(D)

Repair	300 Days			
	Muscle Graft	Nerve Crush	Nerve Graft	N-N Suture
Muscle Graft	*	*	*	*
Nerve Crush	$p< 0.05$	*	*	*
Nerve Graft	N.S.	$p< 0.05$	*	*
N-N Suture	N.S.	N.S.	N.S.	*

Table 8.3.7 - The significance values for the comparison of the form factor of the muscle fibres of the **experimental EDL** after each type of injury and repair. (A) 50 days, (B) 100 days, (C) 200 days and (D) 300 days after operation. (N.S. = no significant difference).

(E)

Repair	50 Days			
	Muscle Graft	Nerve Crush	Nerve Graft	N-N Suture
Muscle Graft	*	*	*	*
Nerve Crush	N.S.	*	*	*
Nerve Graft	N.S.	N.S.	*	*
N-N Suture	N.S.	N.S.	N.S.	*

(F)

Repair	100 Days			
	Muscle Graft	Nerve Crush	Nerve Graft	N-N Suture
Muscle Graft	*	*	*	*
Nerve Crush	N.S.	*	*	*
Nerve Graft	N.S.	N.S.	*	*
N-N Suture	$p< 0.05$	$p< 0.05$	N.S.	*

(G)

Repair	200 Days			
	Muscle Graft	Nerve Crush	Nerve Graft	N-N Suture
Muscle Graft	*	*	*	*
Nerve Crush	$p< 0.01$	*	*	*
Nerve Graft	N.S.	$p< 0.05$	*	*
N-N Suture	N.S.	$p< 0.05$	N.S.	*

(H)

Repair	300 Days			
	Muscle Graft	Nerve Crush	Nerve Graft	N-N Suture
Muscle Graft	*	*	*	*
Nerve Crush	N.S.	*	*	*
Nerve Graft	N.S.	N.S.	*	*
N-N Suture	N.S.	N.S.	N.S.	*

Table 8.3.7 (continued) - The significance values for the comparison of the form factor of the muscle fibres of the **experimental Soleus** after each type of injury and repair. (E) 50 days, (F) 100 days, (G) 200 days and (H) 300 days after operation. (N.S. = no significant difference).

difference either when compared with one another or with contralateral controls. There were no consistent trends to be found in the few groups which did show a significant difference in form factor with respect to time. These results are true both for EDL and soleus muscle fibres.

The standard deviation of the mean form factor remained remarkably constant, averaging 0.04 at 50 and 100 days after operation, 0.05 at 200 days and 0.06 at 300 days.

(c) Distribution and relative proportion of fibre types I and II.

The mean relative number of type I and type II muscle fibres present are illustrated in table 8.3.8 and in figure 8.3.3 (A -P). The pattern of relative abundance of each fibre type present within each muscle was similar at each of the time periods after operation. In the EDL, type II fibres were predominant both in experimental and contralateral control muscles. Type II fibres make up 88.81% of the total number of fibres present in the control muscle and 87.95% in the experimental muscle. Indeed there was no significant difference between the percentage of type I fibres present in the experimental and contralateral control muscles at any time period after a nerve crush injury - see table 8.3.9. After repair by means of a direct epineurial suture the percentage of type I fibres present was not significantly different in a comparison of experimental and contralateral controls with the exception of 200 days after operation ($p<0.05$). After repair by means of the insertion of a graft this difference was significant at the 1% level 200 days after repair by either grafting method and at the 5% level 100 days after repair with a nerve graft and 300 days after repair with a muscle graft. On comparing the

results after each method of injury and repair with one another, there was no difference in the number of type I fibres present at 50 and 300 days after operation - see table 8.3.10. At 200 days after operation the nerve crush group had significantly fewer type I fibres than after any other method of injury and repair. This was significant at the 1% level on comparison with the groups where a graft had been used to repair the nerve and at the 5% level on comparison with the direct epineurial suture group. At 100 days after operation the group in which the animals had been repaired by means of a nerve graft there were significantly more type I fibres than after a nerve crush injury or repair by means of a direct epineurial suture ($p<0.05$ in each case). Although these differences reached statistical significance the graphs clearly showed that the relative proportion of each fibre type present remained remarkably constant for each of the experimental groups. The difference in the relative proportion of each fibre type present after each type of injury and repair did not reach statistical significance in the contralateral control muscles.

The changes in relative proportion of each fibre type present at the different time periods after operation were considered. In the contralateral control muscles there was no significant difference in the relative number of each fibre type present at any time period after operation. In the experimental muscles the difference in the relative proportion of each fibre type present at each of the time periods after operation did not reach statistical significance. This was true for all time periods and all methods of injury and repair with the exception of a significant increase in the number of type I fibres present between 100 and 200 days after repair with

			The percentage of each fibre type present							
			Experimental muscle				Contralateral control muscle			
			Fibre type I		Fibre type II		Fibre type I		Fibre type II	
Duratio	Repair	Muscle	%	S.D	%	S.D	%	S.D	%	S.D
50 days	MG	EDL	22.10	37.06	77.90	40.92	8.10	15.57	91.90	9.83
	NC		38.43	96.24	61.57	105.62	41.03	108.14	58.97	107.40
	NG		13.20	24.87	86.80	21.27	6.85	5.46	93.15	9.26
	NN		9.77	17.99	90.23	31.00	35.67	82.75	64.33	86.89
	MG	Soleus	46.39	31.71	53.61	29.85	98.83	4.72	1.17	2.61
	NC		87.77	21.94	12.23	20.75	83.03	26.89	16.97	28.73
	NG		35.20	23.37	64.80	9.71	86.07	20.34	13.93	18.67
	NN		29.99	46.80	70.01	54.17	39.34	82.77	60.66	85.97
100 days	MG	EDL	6.45	6.60	93.55	12.72	5.61	7.69	94.39	12.18
	NC		4.81	4.56	95.19	4.93	4.01	3.78	95.99	3.70
	NG		9.97	8.17	90.03	12.11	5.18	3.70	94.82	7.09
	NN		5.26	5.45	94.74	11.22	4.00	3.97	96.00	5.55
	MG	Soleus	15.09	10.64	84.91	17.96	53.88	67.23	46.12	73.62
	NC		75.31	85.35	24.69	84.03	84.09	18.53	15.91	17.22
	NG		24.29	79.28	75.71	84.21	70.53	46.05	29.47	56.76
	NN		34.03	20.48	65.97	14.94	84.54	15.06	15.46	9.13
200 days	MG	EDL	13.24	8.32	86.76	15.26	4.47	2.97	95.53	6.76
	NC		5.19	6.54	94.81	10.77	3.82	3.94	96.18	5.55
	NG		12.45	6.12	87.55	10.03	5.96	7.66	94.04	9.31
	NN		12.04	12.22	87.96	10.26	6.57	3.29	93.43	7.13
	MG	Soleus	31.56	7.92	68.44	6.76	98.46	7.14	1.54	3.71
	NC		94.99	8.73	5.01	5.72	96.00	13.24	4.00	12.26
	NG		20.02	26.03	79.98	30.19	81.27	86.42	18.73	83.86
	NN		19.18	10.56	80.82	17.53	97.42	8.70	2.58	5.22
300 days	MG	EDL	8.84	10.97	91.16	7.60	3.54	4.16	96.46	7.26
	NC		9.38	11.01	90.62	40.92	33.92	4.10	66.08	4.76
	NG		10.61	11.30	89.39	17.17	6.12	6.22	93.88	7.60
	NN		10.95	20.28	89.05	17.46	4.10	7.82	95.90	13.55
	MG	Soleus	18.17	13.22	81.83	13.77	96.51	6.77	3.49	6.50
	NC		97.18	3.05	2.82	5.26	96.65	9.57	3.35	4.42
	NG		26.84	40.24	73.16	24.09	91.32	13.42	8.68	11.99
	NN		19.37	18.69	80.63	23.70	88.40	12.29	11.60	15.61

Table 8.3.8 - The relative proportion (%) of type I and type II fibres making up the experimental and contralateral control EDL and soleus muscles after each type of injury and repair and at each time period after operation. (MG = muscle graft, NC = nerve crush, NG = nerve graft, NN = nerve to nerve suture).

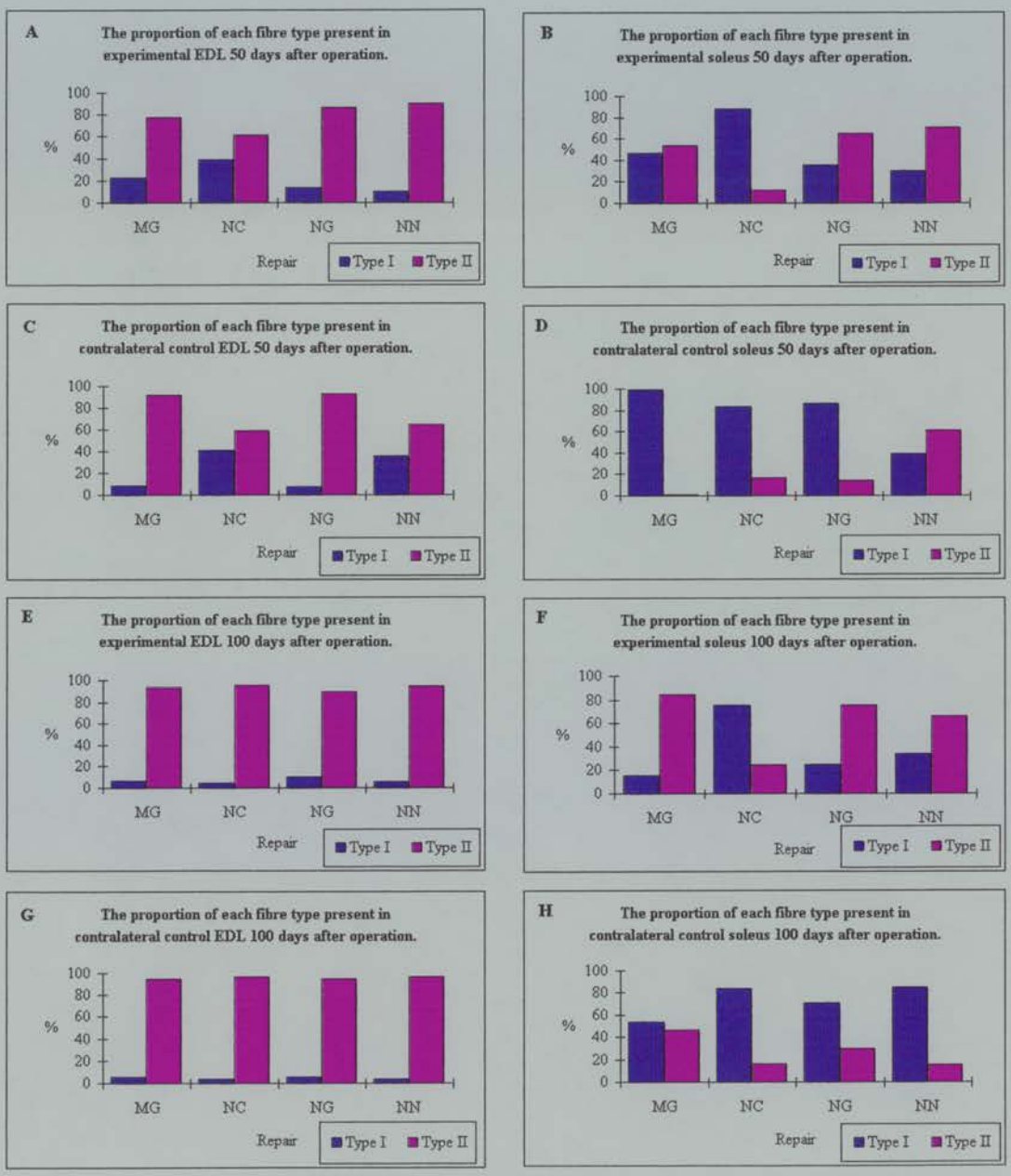

Figure 8.3.3 (A - H) - The relative proportion (%) of type I and type II fibres making up the experimental and contralateral control EDL and soleus muscles after each type of injury and repair. (A) to (D) 50 days after operation and (E) to (H) 100 days after operation. (MG = muscle graft, NC = nerve crush, NG = nerve graft, NN = direct epineurial suture).

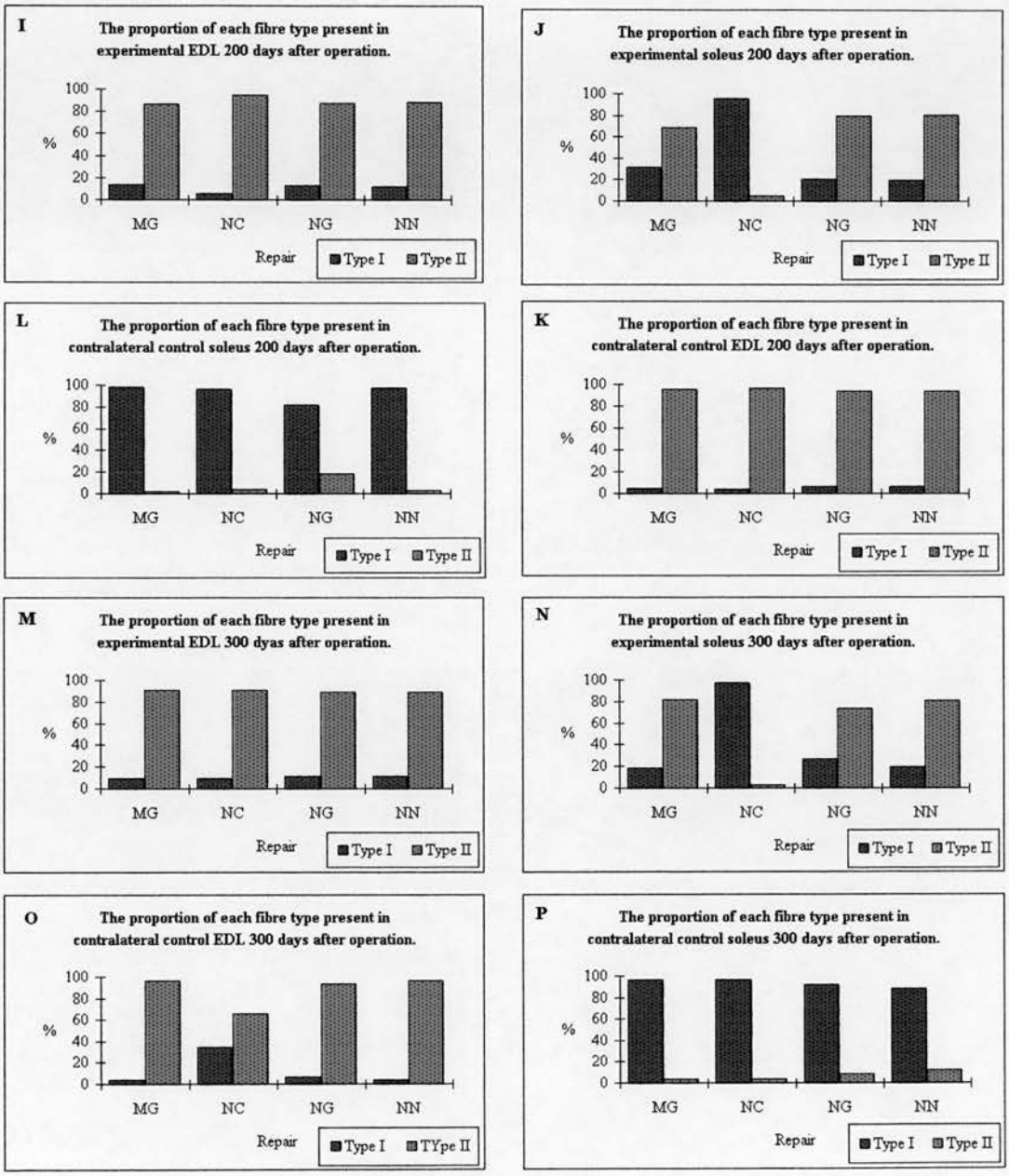

Figure 8.3.3 (I - P) - The relative proportion (%) of type I and type II fibres making up the experimental and contralateral control EDL and soleus muscles after each type of injury and repair. (I) to (L) 200 days after operation and (M) to (P) 300 days after operation. (MG = muscle graft, NC = nerve crush, NG = nerve graft, NN = direct epineurial suture).

Repair	Muscle	Days after operation			
		50	100	200	300
Muscle Graft	EDL	N.S.	N.S.	$p<0.01$	$p< 0.05$
	Soleus	$p<0.001$	$p< 0.05$	$p<0.001$	$p<0.001$
Nerve Crush	EDL	N.S.	N.S.	N.S.	N.S.
	Soleus	N.S.	N.S.	N.S.	N.S.
Nerve Graft	EDL	N.S.	$p< 0.05$	$p<0.01$	N.S.
	Soleus	$p<0.001$	$p< 0.05$	$p< 0.05$	$p<0.001$
N-N Suture	EDL	N.S.	N.S.	$p< 0.05$	N.S.
	Soleus	N.S.	$p<0.001$	$p<0.001$	$p<0.001$

Table 8.3.9 - The significance values for the comparison of the percentage of type I fibres present in the experimental and contralateral control muscles, after each type of injury and repair and at each time period after operation. (N.S. = no significant difference).

(A)

Repair	50 Days			
	Muscle Graft	Nerve Crush	Nerve Graft	N-N Suture
Muscle Graft	*	*	*	*
Nerve Crush	N.S.	*	*	*
Nerve Graft	N.S.	N.S.	*	*
N-N Suture	N.S.	N.S.	N.S.	*

(B)

Repair	100 Days			
	Muscle Graft	Nerve Crush	Nerve Graft	N-N Suture
Muscle Graft	*	*	*	*
Nerve Crush	N.S.	*	*	*
Nerve Graft	N.S.	$p<0.05$	*	*
N-N Suture	N.S.	N.S.	$p<0.05$	*

(C)

Repair	200 Days			
	Muscle Graft	Nerve Crush	Nerve Graft	N-N Suture
Muscle Graft	*	*	*	*
Nerve Crush	$p<0.01$	*	*	*
Nerve Graft	N.S.	$p<0.01$	*	*
N-N Suture	N.S.	$p<0.05$	N.S.	*

(D)

Repair	300 Days			
	Muscle Graft	Nerve Crush	Nerve Graft	N-N Suture
Muscle Graft	*	*	*	*
Nerve Crush	N.S.	*	*	*
Nerve Graft	N.S.	N.S.	*	*
N-N Suture	N.S.	N.S.	N.S.	*

Table 8.3.10 - The significance values for the comparison of the percentage of type I fibres present in the **experimental EDL** after each type of injury and repair. (A) 50 days, (B) 100 days, (C) 200 days and (D) 300 days after operation. (N.S. = no significant difference).

(E)

Repair	50 Days			
	Muscle Graft	Nerve Crush	Nerve Graft	N-N Suture
Muscle Graft	*	*	*	*
Nerve Crush	$p<0.001$	*	*	*
Nerve Graft	N.S.	$p<0.001$	*	*
N-N Suture	N.S.	$p<0.001$	N.S.	*

(F)

Repair	100 Days			
	Muscle Graft	Nerve Crush	Nerve Graft	N-N Suture
Muscle Graft	*	*	*	*
Nerve Crush	$p<0.05$	*	*	*
Nerve Graft	N.S.	$p<0.05$	*	*
N-N Suture	$p<0.01$	$p<0.05$	N.S.	*

(G)

Repair	200 Days			
	Muscle Graft	Nerve Crush	Nerve Graft	N-N Suture
Muscle Graft	*	*	*	*
Nerve Crush	$p<0.001$	*	*	*
Nerve Graft	N.S.	$p<0.001$	*	*
N-N Suture	$p<0.01$	$p<0.001$	N.S.	*

(H)

Repair	300 Days			
	Muscle Graft	Nerve Crush	Nerve Graft	N-N Suture
Muscle Graft	*	*	*	*
Nerve Crush	$p<0.001$	*	*	*
Nerve Graft	N.S.	$p<0.001$	*	*
N-N Suture	N.S.	$p<0.001$	N.S.	*

Table 8.3.10 (continued) - The significance values for the comparison of the percentage of type I fibres present in the **experimental Soleus** after each type of injury and repair. (E) 50 days, (F) 100 days, (G) 200 days and (H) 300 days after operation. (N.S. = no significant difference).

either a muscle graft or direct epineurial suture. This difference was significant at the 5% level in each case.

In the soleus contralateral control muscle, type I fibres were predominant and made up 84.21% of the total number of fibres present. Similarly, in the experimental muscle following denervation due to a crush injury, type I fibres were predominant (88.81%). Indeed there was no significant difference between the percentage of type I fibres present in the experimental and contralateral control muscles at any time period after a nerve crush injury - see table 8.3.9. However after injury and repair by means of a muscle graft, nerve graft or direct epineurial suture the soleus muscle was found to be composed primarily of type II fibres (26.67% type I and 73.33% type II).

After repair by means of a direct epineurial suture the percentage of type I fibres present was significantly less in the experimental muscles as compared to the contralateral controls at 100, 200 and 300 days after operation. This difference was significant at the 0.1% level in each case. After repair by means of the insertion of a graft the experimental soleus had significantly fewer type I fibres at all time periods assessed after operation. This difference was significant at the 0.1% level at all times with the exception of 100 and 200 days after repair by nerve graft and 100 days after repair by a muscle graft ($p<0.05$ in each case). On comparing the results after each method of injury and repair with one another, the number of type I fibres present was significantly greater in the nerve crush group compared to groups in which the nerve had undergone transection and repair. This was significant at the 5% level 100 days after operation and at the 0.1% level at all

other times - see table 8.3.10. On comparing the three methods of repair used after the transection of the nerve the difference in the number of type I fibres present only reached statistical significance on comparing the muscle graft and direct epineurial suture groups 100 and 200 days after operation. This difference was significant at the 1% level in both cases. Thus the relative proportion of each fibre type present varied little between the different methods of repair used after the transection of the nerve. The difference in the relative proportion of each fibre type present after each type of injury and repair did not reach statistical significance in the contralateral control muscles.

The changes in relative proportion of each fibre type present at the different time periods after operation were considered. At 50 and 100 days after operation the contralateral control muscles were made up of 70-75% type I fibres. At 200 and 300 days after operation the contralateral control muscles comprised of >90% type I fibres. The converse was true in the experimental muscles where there was an increase in the relative number of type II fibres from 50 to 300 days. The changes in the number of type I fibres in the contralateral control muscles did not reach statistical significance at any of the time periods after operation. Similarly there was no significant difference in the percentage of type I fibres present after a nerve crush injury at any of the time periods assessed - see table 8.3.11. After repair by means of a direct epineurial suture there was a significant decrease in the percentage of type I fibres present between 100 and 200 days after operation ($p<0.01$) and between 200 and 300 days after operation ($p<0.05$). In the group repaired by a nerve graft there was a significant decrease in the number of type I

fibres present between 50 and 200 days after operation ($p<0.05$). The muscle graft group showed a more marked difference with respect to time with a significant decrease in the number of type I fibres present from 50 to 100 to 200 to 300 days after operation. This difference was significant at the 0.1% level in each case.

The characteristic mosaic pattern of fibre types which is found in normal skeletal muscle was seen in all contralateral control muscles (stained with ATPase) in the current study. Following reinnervation of the muscles which had been denervated by transection of the sciatic nerve, the mosaic pattern was replaced by distinct groups of type I and type II fibres. This was true in all the experimental groups with the exception of the 50 days muscle graft group where the soleus muscle exhibited fibre type grouping but the EDL muscle did not. In the case of reinnervation after a crush injury, the mosaic pattern of fibre types was maintained.

(A)

Duration	Muscle Graft			
	50 Days	100 Days	200 Days	300 Days
50 Days	*	*	*	*
100 Days	N.S.	*	*	*
200 Days	N.S.	$p<0.05$	*	*
300 Days	N.S.	N.S.	N.S.	*

(B)

Duration	Nerve Crush			
	50 Days	100 Days	200 Days	300 Days
50 Days	*	*	*	*
100 Days	N.S	*	*	*
200 Days	N.S	N.S	*	*
300 Days	N.S	N.S	N.S	*

(C)

Duration	Nerve Graft			
	50 Days	100 Days	200 Days	300 Days
50 Days	*	*	*	*
100 Days	N.S	*	*	*
200 Days	N.S	N.S	*	*
300 Days	N.S	N.S	N.S	*

(D)

Duration	N-N Suture			
	50 Days	100 Days	200 Days	300 Days
50 Days	*	*	*	*
100 Days	N.S	*	*	*
200 Days	N.S	$p<0.05$	*	*
300 Days	N.S	N.S	N.S	*

Table 8.3.11 - The significance values for the comparison of the percentage of type I fibres present in the **experimental EDL** at each time period after injury and repair with a (A) muscle graft, (B) nerve crush, (C) nerve graft and (D) nerve to nerve suture. (N.S. = no significant difference).

(E)

Duration	Muscle Graft			
	50 Days	100 Days	200 Days	300 Days
50 Days	*	*	*	*
100 Days	$p<0.01$	*	*	*
200 Days	$p<0.05$	$p<0.001$	*	*
300 Days	$p<0.001$	N.S.	$p<0.001$	*

(F)

Duration	Nerve Crush			
	50 Days	100 Days	200 Days	300 Days
50 Days	*	*	*	*
100 Days	N.S.	*	*	*
200 Days	N.S.	N.S.	*	*
300 Days	N.S.	N.S.	N.S.	*

(G)

Duration	Nerve Graft			
	50 Days	100 Days	200 Days	300 Days
50 Days	*	*	*	*
100 Days	N.S.	*	*	*
200 Days	$p<0.05$	N.S.	*	*
300 Days	N.S.	N.S.	N.S.	*

(H)

Duration	N-N Suture			
	50 Days	100 Days	200 Days	300 Days
50 Days	*	*	*	*
100 Days	N.S.	*	*	*
200 Days	N.S.	$p<0.01$	*	*
300 Days	N.S.	$p<0.05$	N.S.	*

Table 8.3.11 (continued) - The significance values for the comparison of the percentage of type I fibres present in the **experimental Soleus** at each time period after operation after injury and repair with a (E) muscle graft, (F) nerve crush, (G) nerve graft and (H) nerve to nerve suture. (N.S. = no significant difference).

2. Connective tissue.

The volume fraction of connective tissue present within the experimental and contralateral control muscles is shown in table 8.3.12 and in figure 8.3.5 (A - H). The overall mean volume fraction of connective tissue in experimental muscles was 16.06% in EDL and 18.21% in the soleus as compared to the contralateral control levels of 13.26% in the EDL and 14.28% in the soleus. This difference in the overall mean volume fraction of connective tissue between experimental and contralateral control muscles was significant at the 0.1% level for both the EDL and soleus - see table 8.3.13. The amount of connective tissue present in the soleus was significantly greater than in the EDL ($p< 0.001$), this was true both for the experimental and contralateral control muscles. Figure 8.3.4 shows a cross section of a denervated and reinnervated muscle where there has been a proliferation of connective tissue.

Figure 8.3.5 (graphs A - H) clearly demonstrate that the muscles which have undergone denervation and reinnervation consistently have a greater amount of connective tissue than the contralateral control muscles. This is true in all cases with the exception of 200 days after the repairs which involved the insertion of a graft. In both the soleus and the EDL, following grafting, the contralateral control muscle contains a similar amount of connective tissue at 200 days. By 300 days this result has reversed back to the previous trend.

Figure 8.3.6 (graphs A - H) illustrates the difference in the amount of connective tissue between the experimental and contralateral control muscles plotted against time. The graphs clearly illustrate that the overall trend is a

			Mean volume fraction (Vv) connective tissue					
			Experimental muscle			Contralateral control		
Duratio	Repair	Muscle	Vv (%)	S.D.	RSE (%)	Vv (%)	S.D.	RSE (%)
50 days	MG	EDL	22.28	54.84	4.18	17.12	13.79	4.92
	NC		14.32	14.12	5.47	11.20	10.89	6.30
	NG		15.80	28.21	5.16	10.00	9.92	6.71
	NN		21.44	22.35	4.28	19.96	16.54	4.48
	MG	Soleus	24.08	22.78	3.97	14.48	11.28	5.43
	NC		16.48	32.19	5.03	14.24	18.39	5.49
	NG		20.52	9.15	4.40	14.12	7.96	5.51
	NN		19.48	18.43	4.55	12.44	8.58	5.93
100 days	MG	EDL	17.92	5.59	4.79	11.04	3.27	6.35
	NC		17.56	4.09	4.84	11.72	6.11	6.14
	NG		12.52	15.04	5.91	10.32	8.53	6.59
	NN		15.96	18.10	5.13	11.60	7.97	6.17
	MG	Soleus	22.04	18.27	4.21	12.44	8.26	5.93
	NC		18.92	15.50	4.63	15.52	5.81	5.22
	NG		19.20	12.75	4.59	10.80	3.24	6.43
	NN		17.04	16.25	4.93	14.40	16.40	5.45
200 days	MG	EDL	12.92	4.67	5.81	12.96	3.27	5.79
	NC		15.40	5.34	5.24	14.88	9.99	5.35
	NG		13.88	13.79	5.57	13.96	10.57	5.55
	NN		14.44	12.54	5.44	11.28	2.61	6.27
	MG	Soleus	13.20	3.16	5.73	13.36	4.09	5.69
	NC		13.72	10.04	5.61	13.32	8.44	5.70
	NG		14.84	19.98	5.36	15.08	16.32	5.31
	NN		15.40	10.32	5.24	13.28	6.19	5.71
300 days	MG	EDL	16.40	7.25	5.05	15.48	13.16	5.22
	NC		12.96	2.86	5.79	12.36	7.01	5.95
	NG		17.28	7.70	4.89	14.00	6.63	5.54
	NN		16.00	11.16	5.12	14.36	15.19	5.46
	MG	Soleus	21.36	18.73	4.29	17.76	18.69	4.81
	NC		18.52	8.96	4.69	15.88	6.91	5.15
	NG		18.32	23.72	4.72	15.00	12.02	5.32
	NN		18.28	10.74	4.73	16.36	12.76	5.06

Table 8.3.12 - The mean volume fraction (Vv) of connective tissue present in the experimental and contralateral control EDL and soleus muscles after each type of injury and repair and at each time period after operation. (MG = muscle graft, NC = nerve crush, NG = nerve graft, NN = nerve to nerve suture)

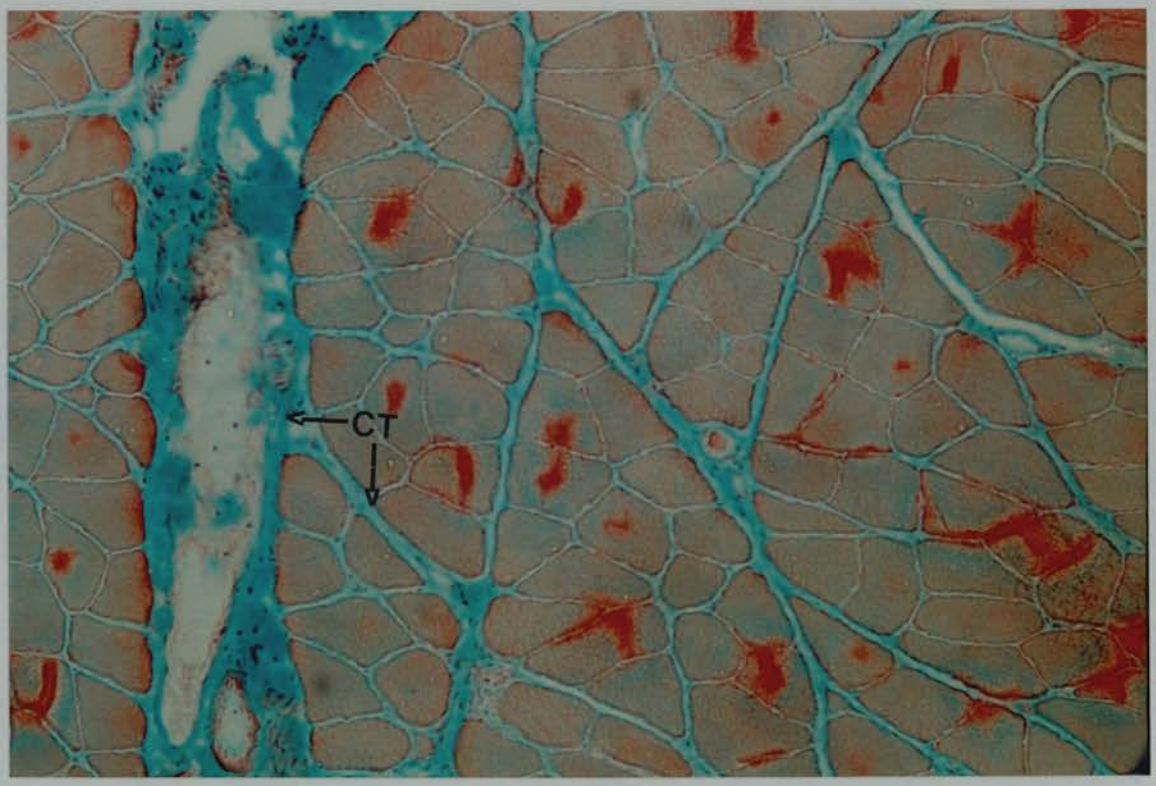

Figure 8.3.4 - Soleus muscle 50 days after the transection of the sciatic nerve and repair with a direct epineurial suture. Note the large areas of connective tissue (CT) which are stained blue/green with Masson's Trichrome. (Magnification x 100).

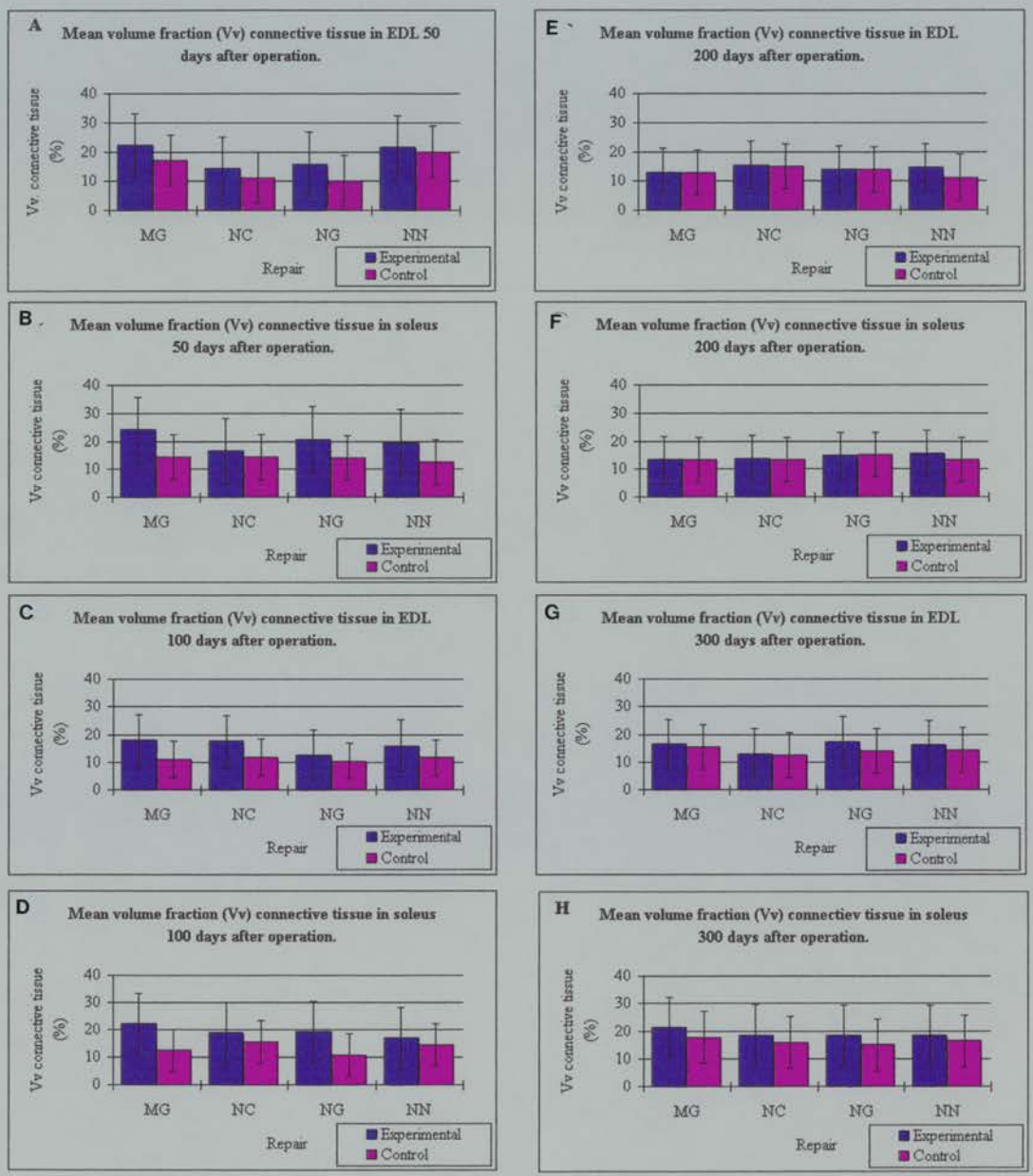

Figure 8.3.5 (A - H) - The mean volume fraction (Vv) of connective tissue present in the experimental and contralateral control EDL and soleus muscles after each method of injury and repair and at each time period after operation. The amount of connective tissue present in EDL and soleus 50 days after operation is shown in graphs (A) and (B), 100 days after operation in graphs (C) and (D), 200 days after operation in graphs (E) and (F) and 300 days after operation in graphs (G) and (H). (MG = muscle graft, NC = nerve crush, NG = nerve graft, NN = direct epineurial suture).

	Muscle	Experimental		Contralateral	
		EDL	Soleus	EDL	Soleus
Experimenta	EDL	*	*	*	*
	Soleus	p<0.001	*	*	*
C. control	EDL	p<0.001	*	*	*
	Soleus	*	p<0.001	p<0.05	*

Table 8.3.13 - The significance values for the comparison of the mean volume fraction (Vv) of connective tissue present in the experimental and contralateral control EDL and soleus, regardless of the type of injury and repair and of the time period after operation. (N.S. = no significant difference).

Repair	Muscle	Days after operation			
		50	100	200	300
Muscle Graft	EDL	N.S.	p< 0.01	N.S.	N.S.
	Soleus	p< 0.01	p< 0.01	N.S.	N.S.
Nerve Crush	EDL	p< 0.05	p< 0.001	N.S.	N.S.
	Soleus	N.S.	p< 0.05	N.S.	p< 0.05
Nerve Graft	EDL	p< 0.05	N.S.	N.S.	p< 0.05
	Soleus	p< 0.01	p< 0.001	N.S.	N.S.
N-N Suture	EDL	N.S.	p< 0.05	p< 0.05	N.S.
	Soleus	p< 0.01	N.S.	N.S.	N.S.

Table 8.3.14 - The significance values for the comparison of the difference in the amount of connective tissue present between the experimental and contralateral control muscles after each type of injury and repair at each time period after operation. (N.S. = no significant difference).

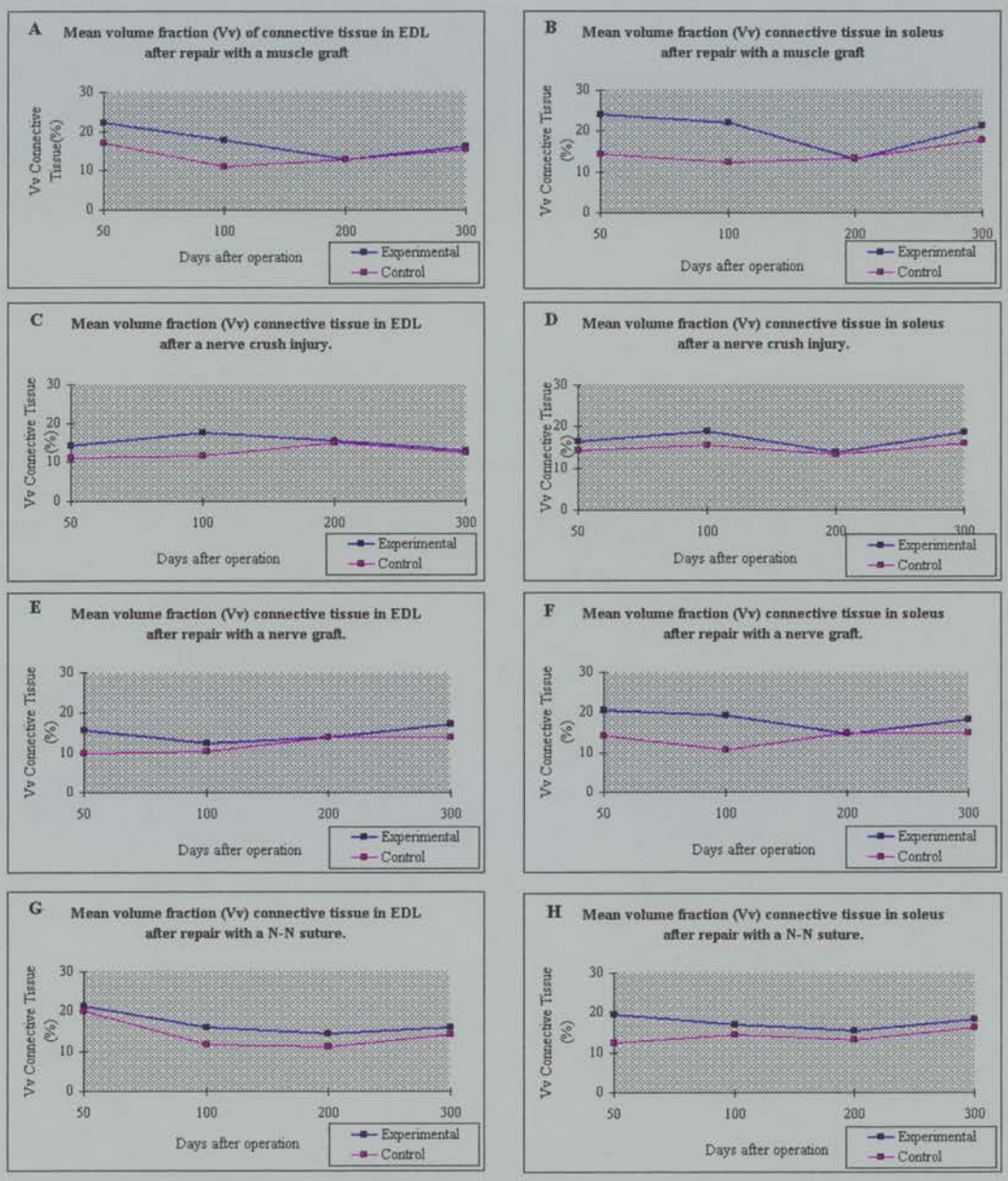

Figure 8.3.6 (A - H) - The mean volume fraction (Vv) of connective tissue present in the experimental and contralateral control EDL and soleus muscles after each method of injury and repair and at each time period after operation. The amount of connective tissue present in EDL and soleus after repair of the sciatic nerve with a muscle graft is shown in graphs (A) and (B), after a nerve crush injury in graphs (C) and (D), after repair with a nerve graft in graphs (E) and (F) and after repair with a direct epineurial suture in graphs (G) and (H).

decrease in the difference between experimental and contralateral control connective tissue levels with time. The difference in the amount of connective tissue present between the experimental and contralateral control muscles tends to be most marked at 50 days after operation. This difference reached statistical significance at the 1% level in the soleus after each of the methods of injury and repair which involved the transection of the nerve - see table 8.3.14. After a nerve crush injury there was no significant difference in the amount of connective tissue in the experimental and contralateral control muscles. In the EDL this difference was significant at the 5% level after a nerve crush injury and after repair with a direct epineurial suture, but failed to reach statistical significance after repair by the insertion of a graft. At 100 days after operation the experimental muscle had a significantly greater amount of connective tissue than the contralateral controls. In the EDL this was true after all repairs with the exception of a nerve graft and in the soleus muscle with the exception of the direct epineurial suture. By 200 days after operation this difference in the amount of connective tissue between experimental and contralateral control muscles had decreased to a level whereby there was little or no difference between the two values. Indeed there was no significant difference between experimental and contralateral control muscle connective tissue content in any group with the exception of the EDL nerve to nerve suture group ($p < 0.05$). By 300 days the values had diverged again with an increase in the amount of connective tissue in the experimental muscles, although this only reached statistical significance in the soleus nerve crush group ($p < 0.05$) and the EDL nerve graft group ($p < 0.05$).

The difference in the amount of connective tissue present in the experimental muscles varied with the repair method. Although this difference reached statistical significance in several cases there was no consistent trend in the connective tissue content of the muscles and the different repair methods - see table 8.3.15.

The graphs clearly show that after repair by means of the insertion of a graft, the soleus muscle was affected by the proliferation of connective tissue much more than the EDL - see table 8.3.16. This difference was most marked at 50 and 100 days after operation, although it only reached statistical significance 100 days after the insertion of a nerve graft ($p < 0.05$). By 200 days after operation the values were very similar and although by 300 days they had diverged again with the soleus showing a greater increase in the amount of connective tissue present, this difference between soleus and EDL did not reach statistical significance. At 50 days after repair by means of a direct epineurial suture, soleus again was affected much more by the proliferation of connective tissue than EDL ($p < 0.05$). At 100, 200 and 300 days after the same repair there was no statistical significance between the amount of connective tissue present in the two muscles. At no time after a crush injury did the difference in connective tissue proliferation between EDL and soleus reach statistical significance. There was no significant difference in the amount of connective tissue present between the contralateral control soleus and EDL muscles at any time.

(A)

Repair	50 Days			
	Muscle Graft	Nerve Crush	Nerve Graft	N-N Suture
Muscle Graft	*	*	*	*
Nerve Crush	N.S.	*	*	*
Nerve Graft	N.S.	N.S.	*	*
N-N Suture	N.S.	$p < 0.05$	N.S.	*

(B)

Repair	100 Days			
	Muscle Graft	Nerve Crush	Nerve Graft	N-N Suture
Muscle Graft	*	*	*	*
Nerve Crush	N.S.	*	*	*
Nerve Graft	$p < 0.05$	$p < 0.01$	*	*
N-N Suture	N.S.	N.S.	N.S.	*

(C)

Repair	200 Days			
	Muscle Graft	Nerve Crush	Nerve Graft	N-N Suture
Muscle Graft	*	*	*	*
Nerve Crush	$p < 0.05$	*	*	*
Nerve Graft	N.S.	N.S.	*	*
N-N Suture	N.S.	N.S.	N.S.	*

(D)

Repair	300 Days			
	Muscle Graft	Nerve Crush	Nerve Graft	N-N Suture
Muscle Graft	*	*	*	*
Nerve Crush	$p < 0.01$	*	*	*
Nerve Graft	N.S.	$p < 0.001$	*	*
N-N Suture	N.S.	$p < 0.05$	N.S.	*

Table 8.3.15 - The significance values for the comparison of the mean volume fraction (Vv) of connective tissue present in the **experimental EDL** after each type of injury and repair. (A) 50 days, (B) 100 days, (C) 200 days and (D) 300 days after operation. (N.S. = no significant difference).

(E)

Repair	50 Days			
	Muscle Graft	Nerve Crush	Nerve Graft	N-N Suture
Muscle Graft	*	*	*	*
Nerve Crush	N.S.	*	*	*
Nerve Graft	N.S.	N.S.	*	*
N-N Suture	N.S.	N.S.	N.S.	*

(F)

Repair	100 Days			
	Muscle Graft	Nerve Crush	Nerve Graft	N-N Suture
Muscle Graft	*	*	*	*
Nerve Crush	N.S.	*	*	*
Nerve Graft	$p< 0.05$	N.S.	*	*
N-N Suture	$p< 0.05$	N.S.	N.S.	*

(G)

Repair	200 Days			
	Muscle Graft	Nerve Crush	Nerve Graft	N-N Suture
Muscle Graft	*	*	*	*
Nerve Crush	N.S.	*	*	*
Nerve Graft	N.S.	N.S.	*	*
N-N Suture	$p< 0.05$	N.S.	N.S.	*

(H)

Repair	300 Days			
	Muscle Graft	Nerve Crush	Nerve Graft	N-N Suture
Muscle Graft	*	*	*	*
Nerve Crush	N.S.	*	*	*
Nerve Graft	N.S.	N.S.	*	*
N-N Suture	N.S.	N.S.	N.S.	*

Table 8.3.15 (continued) - The significance values for the comparison of the mean volume fraction (Vv) of connective tissue present in the **experimental Soleus** after each type of injury and repair. (E) 50 days, (F) 100 days, (G) 200 days and (H) 300 days after operation. (N.S. = no significant difference).

(A)

	Muscle Graft			
	EDL 50 Days	EDL 100days	EDL 200 Days	EDL 300 Days
Soleus 50 days	N.S.	*	*	*
Soleus 100 Days	*	N.S.	*	*
Soleus 200 Days	*	*	N.S.	*
Soleus 300 Days	*	*	*	N.S.

(B)

	Nerve Crush			
	EDL 50 Days	EDL 100days	EDL 200 Days	EDL 300 Days
Soleus 50 days	N.S.	*	*	*
Soleus 100days	*	N.S.	*	*
Soleus 200 Days	*	*	N.S.	*
Soleus 300 Days	*	*	*	N.S.

(C)

	Nerve Graft			
	EDL 50 Days	EDL 100days	EDL 200 Days	EDL 300 Days
Soleus 50 days	N.S.	*	*	*
Soleus 100 Days	*	$p< 0.05$	*	*
Soleus 200 Days	*	*	N.S.	*
Soleus 300 Days	*	*	*	N.S.

(D)

	N-N Suture			
	EDL 50 Days	EDL 100days	EDL 200 Days	EDL 300 Days
Soleus 50 days	$p< 0.05$	*	*	*
Soleus 100 Days	*	N.S.	*	*
Soleus 200 Days	*	*	N.S.	*
Soleus 300 Days	*	*	*	N.S.

Table 8.3.16 - The significance values for the comparison of the mean volume fraction (Vv) of connective tissue in the experimental and contralateral control EDL and soleus muscles at each time period after injury and repair with a (A) muscle graft, (B) nerve crush, (C) nerve graft and (D) nerve to nerve suture. (N.S. = no significant difference).

Discussion

9.1 Alterations in the wet weight of muscle after injury and repair.

On examination of the experimental and contralateral control soleus and EDL muscles it was notable that the muscles excised from the operated leg were generally wasted in appearance compared to the contralateral controls. This was most notable at 50 days after the operation and was negligible by 300 days after operation. This general observation was confirmed by statistical analysis which showed that, at 50 days after operation, the experimental muscle weight was significantly less than the weight of the contralateral control muscles in all but the nerve crush group ($p < 0.05$ in each case). By 300 days after operation the difference between experimental and contralateral control muscle weight was less marked and failed to reach statistical significance with the exception of the group repaired by the insertion of a muscle graft ($p < 0.01$). This decrease in the difference between experimental and contralateral control muscle weight with time is indicative of the reversal of denervation atrophy effects, as a consequence of the reinnervation of the muscle. The reinnervation of the muscle not only results in a decrease in the difference between experimental and contralateral control muscle weights but also an increase in experimental muscle weights with time. The weight of muscles 300 days after injury and repair of the supplying nerve was consistently greater than the weight at 50 days after operation. This difference reached statistical significance in the EDL after all methods of injury and repair ($p < 0.05$ in each case) with the exception of nerve crush injury and in the soleus after repair

with a nerve to nerve suture or a nerve graft ($p < 0.05$ in each case).

The animals which had been injured by means of a nerve crush to the sciatic nerve showed a greater level of recovery in terms of muscle weight than any of the methods of injury which involved the transection of the nerve. 50 days after a nerve crush injury the experimental EDL and soleus had recovered 86.04% and 88.09% of the contralateral control muscle weight respectively. By comparison, the experimental muscles which had undergone transection and repair of the supplying nerve had recovered only between 50.00% and 68.63% of the contralateral control muscle weights. The recovery of the soleus muscle weight to 88.09% of the contralateral control value 50 days after a nerve crush injury compares well with the results of Lowrie and Vrbova (1984) who recorded a recovery of 90.00% 8 weeks after the same injury. By contrast the same study recorded a recovery of only 45.00% of contralateral control muscle weight in the EDL as compared to a recovery of 86.04% found in the current study. This large discrepancy between recovery levels in the EDL between the two studies is likely to be due to a lower than expected level of recovery in the study of Lowrie and Vrbova (1984). The results of Grieve et al (1991) of a recovery of 85% of the contralateral control EDL weight, after transection and repair by means of a muscle graft in the adult rat, are more in line with the results of the current study. Lowrie and Vrbova (1984) concluded that fast muscles such as EDL are permanently affected by nerve injury whereas slow muscles such as soleus make almost a complete recovery. This may be due, in part, to the fact that slow muscles are reinnervated sooner than fast muscles (Jaweed et al 1975). This is in direct

contrast to the results of the current study which found that the soleus muscle tended to show a lower level of recovery of muscle weight than the EDL. Although this only reached a statistical significance in the 50 day nerve graft group and in the 100 and 200 days muscle graft and nerve to nerve suture groups, it was a consistent finding. This finding will be considered later, with respect to the changes in the minimum fibre diameter and the proliferation of connective tissue seen after the injury and repair of the supplying nerve.

On comparing the different methods of injury and repair, the nerve crush group consistently resulted in a greater level of recovery in terms of muscle weight than any of the methods of repair which involve the transection of the nerve. At 50 days after operation the nerve crush group muscles weighed significantly more than those from any other experimental group ($p < 0.05$ in each case). This lesser degree of muscle atrophy after a nerve crush injury to the supplying nerve is likely to be a result of the fact that in a nerve crush injury axonal continuity was maintained. Axonal continuity means that reinnervation can occur more rapidly and more accurately than after injuries which result in the transection of the nerve. As already stated after all methods of injury and repair there is an increase in muscle weight with time as reinnervation occurs. This increase in muscle weight is less marked from 50 to 300 days after a nerve crush injury presumably indicating that the majority of reinnervation occurs before 50 days after operation. This is confirmed by the work of Jaweed et al (1975) who found that reinnervation after a nerve crush injury in the rat occurred 2 -3 weeks after crush. By 300 days after operation the nerve crush group was no longer significantly different from any

other method of injury or repair, indicating that the reinnervation process was similar in all groups by 300 days. The only method of injury and repair which consistently resulted in a significantly lower level of recovery of muscle weight than the nerve crush injury was the insertion of a muscle graft ($p < 0.05$ in each case). This was true at all time periods after operation. The significantly lower muscle weight found after the insertion of a muscle graft implied a greater degree of muscle atrophy and/or a slower rate of reinnervation resulting in a slower recovery of muscle weight. The slower rate of reinnervation may be attributable to the fact that there were two suture lines in the repair. Two suture lines are said to increase the probability of mismatch of regenerating axons and appropriate endoneurial tubes, which results in both inaccurate and delayed reinnervation. This seems an unlikely explanation since the nerve graft also had two suture lines in the repair and yet showed no significant difference in the level of recovery attained compared to the other methods of repair. Since all other factors were consistent between the muscle graft and the nerve graft, it may be assumed that it is the material which forms the body of the graft which causes the difference in the rate of recovery. It is possible that the muscle fibres forming the muscle graft impede the passage of the regenerating axons more so than the nerve graft, resulting in a prolonged period of denervation and hence a greater degree of muscle atrophy.

9.2 Qualitative assessment of muscles.

1. Incidence of muscle fibres with pathological features.

Cazzato (1970) stated that features of chronically denervated muscle consisted of, in descending order of frequency: central nucleation, longitudinal

splitting of muscle fibres, muscle fibre hypertrophy, degenerative changes, random variation in fibre size and interstitial fibrosis. The qualitative assessment of such features in the current study agree with this finding.

Muscle fibres with migrating nuclei and the presence of split muscle fibres were the most common pathological features seen in the current study. These features were present in all experimental muscles and the majority of the contralateral control muscles. The number of these features present in experimental muscles was consistently greater than or equal to the number found in the contralateral control muscles. In 143 out of the 160 experimental muscles, more than 3% of the muscle fibres contained migrating or internal nuclei. By contrast this was the case in only 8 out of the 160 contralateral control muscles. According to Dubowitz (1985) when more than 3% of fibres in a transverse section of a human muscle contain internal nuclei, the muscle is said to have undergone a pathological change. By this measure 89% of experimental muscles have undergone pathological change. Of the 11% of muscles which failed to undergo pathological change, 8% had undergone the less severe injury of a nerve crush rather than the complete transection of the supplying nerve. In all experimental muscles the number of fibres with migrating nuclei was less than 10%, which constitutes a minimal abnormality according to the definitions of Dubowitz (1985).

The migration of the nuclei into the centre of the muscle fibre is thought to precede the longitudinal splitting of the fibre (Banker 1960). Some authors have noted that when a fibre splits, the process often begins at the site of an internal nucleus (Schwartz et al 1976) whilst other studies claim the split usually begins at

the periphery and is directed towards a centrally located nucleus (Jennekens 1982, Swash and Schwartz 1984). There is a general agreement however, that no matter how the splitting process occurs it is usually always associated with the presence of internal nuclei (Dubowitz 1985). Longitudinal splitting of a muscle fibre does not occur in normal muscle except near the myotendonous junction (Bell and Conen 1968, Swash and Schwartz 1984), however it has been seen in a number of neuromuscular diseases and can be induced by training or functional overload (Bell and Conen 1968, Edgerton 1970, Hall Craggs 1970, Salminen and Vihko 1983). Fibre splitting can take the form of a partial split resulting in the forking of individual fibres, a complete split resulting in the formation of two smaller fibres or multiple splits which results in the formation of many small fibres and is often referred to as fragmentation (Schwartz et al 1976, Jennekens 1982). Examples of each of these types of split were seen in the current study. The splitting of muscle fibres was found in 3% to more than 10 % of muscle fibres in all experimental muscles as compared to less than 6% of muscle fibres in contralateral controls. No note was made of the size of the muscle fibres which were split, although it has been noted by some authors that it is a more common feature in large, especially hypertrophied fibres (Schwartz et al 1976). This finding fits in with the fact that central nucleation is also a more common occurrence in hypertrophied fibres (Swash and Schwartz 1984).

It must be considered that in the current study the differences seen between the number of split fibres in the experimental and contralateral control muscles may not be as marked than if an age, sex and weight matched control had been used.

As previously stated, training or functional overload can induce fibre splitting in normal muscles. In the current study the experimental muscle was denervated resulting in temporary loss of function and prolonged weakness. In order to remain mobile, the animals favoured the use of the contralateral limb thus increasing the load on the control muscle. Under the pressure of an increased work load, muscle fibres tend to split (Swash et al 1978, Bradley 1979, Salminen and Vihko 1983, Kakulus et al 1985). Thus although the current study showed a marked increase in the number of split fibres in the experimental muscles, the actual difference between the experimental and normal values may be greater than indicated by this study. The alterations in the numbers of other pathological features present, which are not influenced in this way, will provide a more accurate insight into the degree of postoperative changes in the muscle fibre architecture.

The presence of angulated fibres is a classic feature of denervation. Denervation of a muscle causes the fibres to decrease in size, consequently one or several of the straight sides of the fibre becomes concave and the angles decrease from 120 degrees to less than 90 degrees. This results in the formation of an angulated fibre (Jennekens 1982). In the current study angular fibres were seen in 98% of the experimental muscles as compared to 17% of the control muscles. In each case the number of angular fibres present in the experimental muscles was greater than or equal to the number in the contralateral control muscles. There was no consistency between the presence of angular fibres and the time between injury and repair, although there generally tended to be fewer angular fibres present 200 and 300 days after operation than after 50 and 100 days. The fact that angular fibres remained 300 days after injury and repair suggests that they had not regained

their former size. If the fibres had increased in size, either back to their original size or enlarged owing to hypertrophy, the angulation of the fibre would have been lost. The failure to increase in size may raise doubts as to whether reinnervation of these fibres had occurred, however the fact that the fibres had remained angular and had not become small and rounded suggests that atrophy had not proceeded, therefore reinnervation was likely.

Granular fibres are indicative of degeneration (Dubowitz 1985). They have a coarsely granular appearance which stains blue with haematoxylin and eosin and red with a modified Gomori's trichrome stain (ragged red fibres). In the current study granular fibres were found in 98% of experimental muscles and in 12.5% contralateral controls. In each case the number of angular fibres present in the experimental muscles was greater than or equal to the number in the contralateral control muscles. There was no consistency between the presence of granular fibres and the time between injury and repair, although there generally tended to be fewer granular fibres present at 200 and 300 days after operation than after 50 and 100 days.

Moth eaten fibres are so named because denervation causes a disruption of the intermyofibrillar network which produces patchy staining and a ragged appearance. In the current study moth eaten fibres were found in 93.75% of experimental muscles and in 11% contralateral controls. In each case the number of moth eaten fibres present in the experimental muscles were greater than or equal to the number in the contralateral control muscles. As with the other pathological features there was no consistency between the presence of moth eaten fibres and

the time between injury and repair. Again, as with the other pathological features, moth eaten fibres were more prevalent at 50 and 100 days after operation than after 200 and 300 days.

Circular or whorled fibres are caused by the disorientation of the longitudinal pattern of the myofibrils, which results in the characteristic circular appearance of the fibres (Dubowitz 1985). In the current study circular fibres were found in 11% of experimental muscles and in 7.8 % of the contralateral control muscles. They were often seen in aggregations of several fibres rather than individually, a fact that was also noticed by Dubowitz (1985). Circular fibres were usually very much larger than the other fibres. The number present was not consistently greater in experimental muscles which is in direct contrast to the other pathological features. The number of circular fibres present was greater in the experimental muscles in three cases, greater in contralateral control muscles in three cases and equal in all other cases. There was no consistency between the number of circular fibres present and the time between injury and repair. The presence of circular fibres was thought to be a feature of reinnervation rather than denervation (Dubowitz 1967) however since they have not been reported in other reinnervation experiments this is now in some doubt (Karpati and Engel 1968c, Jennekens 1982) Their presence is known to be non specific and to occur in various dystrophies, chronic neuropathies and other disorders (Jennekens 1982, Dubowitz 1985).

Comparing the different methods of injury and repair, the nerve crush injury groups were consistently associated with fewer fibres with pathological features than any of the injuries which involved the transection of the nerve. This is most

likely to be related to the fact that the nerve crush injury was a less severe injury in which neural alignement was maintained and therefore reinnervation of the muscle was more rapid and more accurate. The fact that reinnervation occurred more quickly means that denervation effects on the muscle were less advanced and were therefore more readily reversed by reinnervation. After the repair of a transected nerve using a muscle graft there was generally a greater number of fibres with pathological features than after a nerve to nerve suture, which in turn resulted in a greater number of fibres with pathological features than after a nerve graft. There was general trend towards a decrease in the number of pathological features by 300 days after operation although this was by no means consistent. The number of fibres in the EDL with pathological features was consistently less than or equal to the number present in the soleus. This was true both for experimental and contralateral control muscles. This may have been due to the fact that the soleus muscles are made up primarily of type I fibres which are said to be more susceptible than type II (Jaweed et al 1975). The different reactions of type I and type II muscle fibres will be considered later see chapter 9.3.

2. Histochemical fibre types.

(a) Fibre type grouping

Fibre type grouping is discussed as part of the quantitative assessment of fibre types under 'Distribution and relative proportion of fibre types I and II.'

(b) Fibre type predominance.

Fibre type predominance is discussed as part of the quantitative assessment of fibre types under 'Distribution and relative proportion of fibre types I and II.'

3. Size range of muscle fibres and fascicles.

The qualitative assessment of the size range of the muscle fibres resulted in extremely variable results. There were no consistencies between the experimental groups, time periods after operation or even between contralateral control muscles. It was decided that the size of the muscle fibres could be assessed much more accurately using quantitative means (see below).

9.3 Quantitative assessment of muscles.

1. Measurement of muscle fibres.

(a) Minimum diameter of muscle fibres.

The size of the muscle fibres was assessed by measuring the minimum fibre diameter. The minimum fibre diameter is defined as being the greatest distance between the opposite sides of the narrowest aspect of the muscle fibre (Aherne and Dunhill 1982). It is one of the most accurate methods of assessing fibre size since minimum fibre diameter measurements are least affected by variations in the plane of section away from the true transverse plane (Swash and Schwartz 1984, Dubowitz 1985).

One of the fundamental properties of muscle fibres is that under the stress of repetitive and sustained work they will increase in size, *i.e.* work induced hypertrophy (Dubowitz 1985). The converse is also true, in that when muscle fibres are not active for a prolonged period they will decrease in size, i.e. disuse atrophy. Both atrophied and hypertrophied fibres are a common occurrence in muscles which have undergone denervation and reinnervation. Muscle fibre atrophy is a consequence of denervation and arises as a result of the muscles being

deprived of the trophic influence of their innervation and as the result of disuse of the muscle (Dubowitz 1985). Fibre splitting has also been classified as muscle fibre atrophy (Swash and Schwartz 1984), however although this process causes a decrease in the fibre diameter, it is debatable whether it can be classed as true atrophy. Muscle fibre hypertrophy commonly occurs in partially denervated muscles, or in the case of complete denervation, in those muscle fibres which are reinnervated first. In such muscles, where there has been a loss of functioning muscle fibres, hypertrophy is a compensatory response to the increased work load imposed on the surviving healthy muscle fibres (Swash and Schwartz 1977).

The degree of muscle fibre atrophy and the speed of onset is variable between species, individuals, muscles and fibre types. Atrophy is evident in human muscles within a few weeks of denervation whereas in the cat there is no evidence until after more than one month of denervation (Adams 1975). In Sprague Dawley rats, the soleus muscle fibres had atrophied to 20% of control values 3 weeks after denervation (Stonnington and Engel 1973) whereas in the Australian opossum atrophy had only reached 30% of control values by 60 days after denervation (Sunderland and Ray 1950). In the current study muscles fibre atrophy never exceeded 50%, indeed atrophy was less than 20% in more than 90% of type I fibres and in more than 70% of type II fibres. Even this level of muscle fibre atrophy is likely to have been exaggerated since the contralateral muscles were used as controls. The contralateral control muscles were likely to have undergone compensatory hypertrophy, as a consequence of the increased work load which resulted from the inability of the denervated limb to support the weight of the

animal. Thus, if the contralateral control muscles had hypertrophied, this would exaggerate any apparent atrophy in experimental muscles with which they were compared. This problem could have been avoided with the use of age, sex and weight matched controls.

The minimal levels of atrophy seen in the current study, combined with the fact that there was a minimal increase in the minimum diameter of muscle fibres with time, indicates that the vast majority of reinnervation which occurred after peripheral nerve repair did so before 50 days after operation. This is in agreement with the results of other studies which show that reinnervation occurs 2 - 3 weeks after a nerve crush injury in the rat (Jaweed et al 1975) and 4 - 7 weeks after a nerve transection injury, followed by repair, in the rat (Glasby et al 1986).

In the contralateral control soleus muscle, in the current study, the mean minimum diameter of type I fibres tended to be greater than that of type II fibres. This is in agreement with the results of Jaweed (1975) - in the rat, Herbison et al (1979) - in the rat and Kennedy (1987) - in the mouse. After injury and repair of the sciatic nerve, type I fibres remained greater in diameter than type II fibres. This was true in both the nerve crush and nerve transection groups. This is in agreement with the results of Kennedy (1987) 3 weeks after a nerve crush injury in the mouse and the results of Jaweed (1975) 6 weeks after a nerve crush injury in the rat (although not at 3 weeks). However, it is in disagreement with the results of Kennedy (1987) after the transection of the nerve. The study by Kennedy (1987) only observed the changes up to 3 weeks after denervation, if the study had been continued to 6 weeks after operation the results may well have matched the results

of both the current study and of Jaweed (1975).

In the contralateral control EDL, in the current study, the mean minimum diameter of type I fibres tended to be less than type II fibres. This is in agreement with results of Niederle and Mayr (1978) in the rat and Kennedy (1987) in the mouse. After injury and repair to the sciatic nerve, type II fibres remained greater in diameter than type I fibres. This was true in both the nerve crush and nerve transection groups. This is in agreement with Kennedy (1987) who also found no significant difference in the mean minimum diameter of the different fibre types between those muscles denervated by means of a crush injury and those denervated by nerve transection in the mouse. However it is contrary to the findings of Niederle and Mayr (1978) who found that type II fibres atrophied whilst type I fibres remained unchanged in the rat. This resulted in type I fibres having a greater minimum diameter than type II fibres 42 days after denervation. In the current study there was no consistency in the changes in the mean minimum diameter of type I and type II fibres of the soleus and EDL when considered with respect to time or method of injury and repair.

Opinion in the literature is very varied on the subject of the rate and extent of atrophy of different muscle fibre types. Selective type II atrophy is a common feature in myopathies, disuse atrophy, after strokes and in Parkinson's disease (Swash and Schwartz 1984). There was a generally held assumption that transection of a peripheral nerve in the adult animal also caused the preferential atrophy of type II muscle fibres (Jaweed 1975). Indeed this was confirmed by the findings of Engel et al (1966), Karpati and Engel (1968) and Wuerker and Bodley

(1973). This finding has been contradicted by other authors who found type I fibres to atrophy more than type II (Kennedy 1987). Contrary to both these opinions, there is a third group who believe the two fibre types to atrophy equally (Romanul and Hogan 1965, Herbison et al 1973, Tomanek and Lund 1973, Jaweed et al 1975). The findings of the current study agree with the findings of this latter group.

From an overview of the literature it would appear that some of this disagreement has arisen from the different reactions of the two muscle fibre types depending on the muscle in which they are situated, i.e. the reaction of fast twitch muscle fibres in a fast twitch muscle may be different to their reaction in a slow twitch muscle (Jaweed et al 1975). Jaweed et al 1975 reported that type I (slow twitch) fibres atrophied more in a slow twitch muscle such as soleus and type II (fast twitch) fibres atrophied more in fast twitch muscle such as plantaris. In the current study the mean minimum fibre diameter was generally greater in the soleus muscle fibres than in the EDL. This was most marked in the type I fibres when compared to type II fibres. However although the diameter measurements were larger in the soleus muscle there was little or no difference in the degree of atrophy following denervation of the muscle.

In normal muscle, the standard deviation of the mean minimum fibre diameter should be 0.25 times the value of the mean (Dubowitz 1985). This denotes a normal variability in muscle fibre size. There was a total of 2 out of the 32 contralateral groups which failed to meet this requirement and a total of 5 out of 32 of the experimental groups. Thus 94% of contralateral control muscles and 86% of experimental muscles fell within the normal variation in muscle fibre size.

Thus although the mean minimum fibre diameter was less in the experimental muscles than the contralateral control, there was no abnormal variability in muscle fibre size for the vast majority of the muscles. It may be concluded from this that the muscle fibres had all undergone a similar degree of atrophy during the denervation/reinnervation process.

(b) Form Factor

Muscle fibres, when seen in cross section, are generally polygonal in shape. The characteristic polygonal shape is the result of the mutual compression of the fibres which are closely packed together within the perimysium. When a muscle is denervated, the fibres atrophy resulting in an increase in the free space within the muscle. This increase in free space reduces the pressure the fibres exert on one another and hence allows the fibres to assume a more circular shape. The more circular a fibre is in cross section the nearer the form factor is to 1. In the current study there was no significant difference between the form factors of experimental and contralateral control muscle fibres. When considering the form factor of the muscle fibres after each of the methods of injury and repair, the vast majority of groups showed no significant difference. In the very few cases where there was a significant difference between the repair methods there was no consistency between groups. This was also true when considering the form factor of muscle fibres with respect to time. This was true both for the soleus and the EDL. Hence in the current study the denervation and reinnervation of the muscle fibres had a negligible effect on the shape of the fibres. This result suggests that the various methods of repair had resulted in the successful reinnervation of the muscle, either

preventing or reversing atrophy and hence allowing the muscle fibres to retain their polygonal shape. Kennedy (1987) showed that in partially denervated soleus muscle there was a very large range in fibre shapes resulting in a large standard deviation of the mean shape factor. This was explained by the fact that the innervated muscle fibres hypertrophied, squeezing the denervated muscle fibres resulting in angular fibres. In the current study there was very little variation in either the mean form factor or the standard deviation. This suggested that all signs of atrophy and hypertrophy caused by the denervation and reinnervation of the muscle fibres had passed before the first postoperative observation at 50 days.

(c) Distribution and relative proportion of fibre types I and II.

The nature of any individual muscle depends on the relative proportions of type I and type II fibres which it contains. Type I fibres have a low activity of actomyosin ATPase. Since the ATPase activity of a fibre correlates with its speed of contraction (Barany 1967) this means that any muscle which is predominantly composed of type I fibres will be a slow twitch muscle. The converse is true of fast twitch muscles. The relative number of each fibre type present varies between different muscles and is also dependent on the age and species of the individual. Taking these factors into consideration there is a general agreement in the literature that the normal soleus muscle of the adult rat comprises 75 - 80% type I fibres and 20 - 25 % type II fibres (Barnarard et al 1971, Kugelberg 1973, Ariano et al 1973, Jaweed et al 1975, Pullen et al 1977, Shiaffino et al 1979, Narasuwa 1985). The normal EDL muscle of the adult rat comprises 3 - 10% type I fibres and 90 - 97% type II fibres (Ariano et al 1973, Pullen et al 1977, Nierdele and

Meyer 1978). The results of the current study lie slightly outwith these predicted ranges, with the rat soleus muscle being made up of 84.21% type I and 15.79% type II fibres and the EDL muscle 11.19% type I and 88.81% type II. The results of the current study indicate that there are marginally more type I fibres present in both the EDL and soleus than the other studies suggest. The consistent labelling of a greater number of type I fibres suggests that this discrepancy was due to the staining technique employed. The staining technique used in the current study may be either more sensitive to type I fibres or less sensitive to type II fibres than the techniques used by other authors. This result illustrates the need for caution when comparing the results of studies which involve the use of different staining techniques, although it is not a problem in this comparative study.

Following the injury and subsequent repair of the peripheral nerve, there was little or no difference in the relative proportion of each fibre type present in the EDL. Type II fibres made up 88.81% of the contralateral control muscle fibres as compared to 87.95% of the experimental muscle. This trend was consistent at all times after operation. Similarly, Kennedy (1987) found no significant difference between control and denervated EDL in the mouse (type II fibres making up 99.35% in controls and 99.38% in experimental muscles). The method of denervation, i.e. nerve cut or crush, had no effect on the relative proportions of fibre types present in the EDL, with 85.54% type II fibres after nerve crush and 88.76% after nerve cut. Kennedy (1987) found similar results in the mouse with a difference of only 0.17% in the number of type II fibres present after denervation caused by a crush injury and caused by severance of the nerve. Jaweed et al (1975)

studied the effect of denervation on the plantaris of the rat, a fast muscle like the EDL, and found similar results, 89% type II fibres after nerve cut, 90% type II fibres after nerve crush and 90% type II fibres in the control muscle.

After a crush injury to the sciatic nerve the soleus muscle retained approximately the same relative proportions of fibre types as the contralateral controls (84.21% type I fibres and 15.79% type II fibres in contralateral controls and 88.81% type I and 11.19% type II in experimental muscles). After severance of the nerve and repair by means of a nerve graft, muscle graft or direct epineurial suture there was a significant increase in the number of type II fibres present in the soleus muscle ($p < 0.001$ at 300 days after operation in each case). Indeed the muscle changed from being predominantly slow in the control muscle (84.21% type I fibres) to predominantly fast (26.67% type I fibres). A similar phenomenon was noted in the guinea pig by Karpati and Engel (1968), in the rat by Gutmann et al (1972), Tomaneck and Lund (1973), Jaweed et al (1975) and in the mouse by McLachlan (1983). In both the experimental and contralateral control muscles there was a change in the relative number of each fibre type present with time. In the contralateral control muscles the relative number of type I fibres present increased with time whilst in the experimental muscles there was an increase in the number of type II fibres present.

There has been a wide variety of suggestions put forward as to the reason behind this transformation of the soleus muscle from slow to fast. Jaweed (1975) proposed that this was due to disuse or inactivity caused by the denervation. Vrbova (1963), Fischback (1969) and Buller (1972) demonstrated that a slow

muscle could be changed to a fast muscle by atrophic processes caused by upper motor neuron lesions, immobilization and tenotomy respectively. Similarly, severance of the sciatic nerve in the work of Gutmann et al (1972) and Tomaneck and Lund (1973) resulted in a similar change. This change was again attributed to the disuse phenomenon. It is now widely accepted that the transformation of the soleus muscle from slow to fast, after nerve section, is due to type I fibres becoming innervated by type II neurones (Buller et al 1960, Dubowitz 1967, Karpati and Engel 1968c, Jennekens 1982). Since motor units are uniform in their functional properties (Dubowitz 1985, Slater and Harris 1988) and histochemical characteristics (Edstrom and Kugelberg 1968, Mayer and Doyle 1970, Burke et al 1971, Kugelberg 1973, Pette and Vrbova 1985), such reinnervation results in the transformation of the type I fibres into type II fibres. Consequently the soleus muscle contracts more quickly due to changes in the myosin isoforms, metabolic enzyme profiles and the extent of vascularization (Pette and Vrbova 1985). Buller et al (1960, 1962, 1965 and 1969) proposed two possible mechanisms of neural influence on the speed of contraction. The first hypothesis being that the neural influence on speed is related to the pattern of motor nerve impulses and the second hypothesis being the production of specific trophic substances by the motor nerve which is passed on to the muscle fibre that it innervates. Although several authors (Eccles and Buller 1962, Salmons and Vrbova 1969, Fex 1969, Fex and Sonesson 1970) have investigated these theories neither has been proved conclusively. The uniformity of the motor unit is always maintained by the conversion of the muscle fibre to match the supplying motor nerve - the motoneurones retain their

characteristic type specific properties regardless of any alterations in their target muscle (Kuno et al 1974).

The transformation of fast and slow muscle fibres which occurs after the injury and repair of a peripheral nerve, not only changes the relative number of each fibre type present but also changes their spatial distribution. In a normal muscle the fibres belonging to different motor units are usually intermingled (Edstrom and Kugelberg 1968, Mayer and Doyle 1970, Kugelberg 1973 and 1979, Dubowitz 1985). When histochemical stains are applied, this results in a mosaic like pattern of enzyme activity (Jennekens 1982). The mosaic pattern approximately conforms to a random distribution (Swash and Schwartz 1984). All contralateral control muscles in the current study were found to have this characteristic mosaic pattern when stained with ATPase. Following the reinnervation of muscles after the transection of the sciatic nerve, the mosaic pattern had been replaced by distinct groups of fibres of the same histochemical type. This fibre type grouping was first described by Brooke and Engel (1966) and was also seen after the section and suture of a motor nerve by Karpati and Engel (1968c) and after cross reinnervation by Dubowitz (1967a) and Romanul and Van der Meulen (1967). The grouping of the motor units is the result of collateral sprouting of the regenerating nerve. Only a percentage of the regenerating nerve fibres will reach the distal portion of the nerve and yet many, if not all, of the muscle fibres will be reinnervated (Jennekens 1982). The regenerating nerves tend to throw out collateral sprouts which synapse with neighbouring muscle fibres, consequently muscle fibres of a motor unit are grouped together instead of being

intermingled with fibres of another (Jennekens 1982). Kugelberg (1973) pointed out that type groups are often restricted to one fascicle, which is probably attributable to the connective tissue which separates the fascicles acting as a barrier to the outgrowing sprouts. In the current study all the groups of fibres seen within the experimental EDL were restricted to one fascicle as Kugelberg described. In the soleus muscle the smaller groups of fibres (small group 12-15 fibres) were also contained in this way, however many of the experimental soleus muscles contained such large groups of type II fibres that they constituted more than half of the fibres in a section. Such large groups obviously could not be restricted to one fascicle. In the current study type grouping was seen in all experimental groups which involved the transection of the nerve with the exception of 50 days after the insertion of a muscle graft. In this case type grouping was seen in the soleus muscle but not in the EDL. After the nerve crush injury there was no such evidence of fibre type grouping. It is likely that since a nerve crush is a less severe injury which maintains neural alignment, the number of regenerating axons which successfully reached and reinnervated their target muscle fibres was much greater than after the transection of the nerve. Consequently there is no need for the regenerating axons to throw out the collateral sprouts which result in fibre type grouping. The fibre type grouping was seen at all time periods after operation. This finding was not in agreement with the results of Warszanski et al (1975) who found that fibre type grouping does not appear earlier than 6 weeks after peripheral nerve injury (crush in rat).

2. Connective tissue.

The volume fraction of connective tissue in the EDL and soleus was assessed at each of the 4 time periods after each of the nerve repair operations. The overall mean volume fraction of 13.26% in the EDL and 14.28% in the soleus of contralateral control animals and 16.06% in the EDL and 18.21% in the soleus of experimental animals compares very well with the results of Grieve et al (1991), although they are somewhat higher than the values quoted by Birzgalis 1979 (6.4% EDL and 9.7% soleus). This difference is possibly due to the fact that the work of Birzgalis was based on young and undernourished animals. Grieve et al (1991) stated that there was an increase in connective tissue of 2% in adults and 4% in elderly rats following repair by means of a muscle graft, these results compare well with our results of an overall increase of 3.23% following the same repair.

In the current study, muscles which have undergone denervation and reinnervation consistently contained a greater level of connective tissue than the contralateral controls. Opinion on this matter is very mixed in the literature. Tower et al (1935) - in the cat, Bowden and Gutmann (1944) - in the human, Gutmann and Young (1944) - in the rabbit, Sunderland and Ray (1950) - in the opossum and Grieve et al (1991) - in the rat all reported a similar increase in connective tissue after peripheral nerve injury, however Willard and Graw (1924) - in the mouse and Jakubec-Puka and Laskowska-Bosek (1974) - in the rat noted no such proliferation. The elevated levels of connective tissue in the experimental muscles was probably related to the degree of atrophy of the denervated muscle fibres. As the muscle atrophies, spaces form between the muscle fibres. The formation of

these spaces is thought to stimulate fibroblasts into activity and hence the amount of connective tissue increases (Bowden and Gutmann 1944). Indeed it was noted by Sunderland and Ray (1950) that the proliferation of connective tissue was always preceded by a marked increase in the number of fibroblasts present. It is widely believed that the cross sectional area of muscle fibres lost through atrophy is replaced by the proliferation of connective tissue, however Dubowitz (1985) suggested that the proliferation plays a more active role in the pathogenesis of the dystrophic process.

After peripheral nerve injury there is a period where the muscle will undergo the classic changes associated with denervation, such as decrease in fibre diameter, increase in connective tissue etc. Many of these changes will gradually be reversed as the regenerating nerve fibres reach the muscle and reinnervation takes place. By 50 days after operation, reinnervation will have begun but the muscle will still show signs of denervation. With time, the atrophied fibres will increase in size hence the muscle tissue will become relatively more abundant than the connective tissue. It is for this reason that the difference in the amount of connective tissue present in experimental and contralateral control muscles was most marked at 50 days after operation and then decreased with time.

In general, the soleus muscle tended to have a greater increase in connective tissue content than the EDL. This may be due to the preferential atrophy of type I fibres following denervation of the muscle (Bajusz 1964 (in rat and mouse), Riley and Allin 1973 (in the cat), Jaweed 1975 (in the rat)). There is disagreement in the literature regarding the preferential atrophy theory with some authors claiming that

type I fibres atrophy to a greater degree (Bajusz 1964 (in rat and mouse), Riley and Allin 1973 (in the cat), Jaweed 1975 (in the rat)) whilst others believe that type II fibres are more likely to atrophy first (Karpati and Engel 1968a, Niederle and Mayr 1978 (in rat)). It would appear that the extent and the rate of atrophy of different fibre types is variable among different muscles and is dependent on the duration of denervation (Niederle and Mayr 1978a, Jaweed 1975). There is however general agreement that type I fibre atrophy is more pronounced in slow muscles than in fast (Tomaneck and Lund 1973, Jaweed et al 1975). Since the soleus is a slow muscle then it is likely that there is indeed preferential denervation of type I fibres. Such preferential denervation will obviously have a marked effect on the soleus muscle as it consists of 95% type I fibres. The proliferation of connective tissue tends to increase as atrophy of the muscle fibres advances and so the preferential atrophy of type I fibres could cause the increase in connective tissue found in this study. The results of Pluskal and Pennington (1976) and Cullen and Pluskal (1977) agree with these findings that in the early stages of denervation changes in soleus are greater than in EDL.

The proliferation of connective tissue tends to increase as atrophy of the muscle fibres advances. These two factors together can make re-connection with regenerating nerve fibres difficult if not impossible (Bowden and Gutmann 1944). The connective tissue interferes with the connection between the Schwann cell tubes of the regenerating nerve and the old motor end plates. It is thought that the new collagen blocks the Schwann cell tubes (Gutmann and Young 1944). This blockage is partial initially and so the returning flow of axoplasm is forced into the

a few remaining open channels to reach the old end plate. As the increase in connective tissue progresses the channel to the old end plate becomes blocked and so the flow is forced to deviate along the fibres. If these escaped fibres make contact with the sarcoplasm of the muscle fibre then new motor end plates will be formed. However the chances of making contact at all decreases as the muscle fibres shrink and the collagen around them thickens. Moreover it becomes progressively less likely that a nerve will reinnervate its original set of muscle fibres. This diversion of the axoplasm flow from the original end plate along the fibres produces the characteristic pattern of reinnervation (Gutmann and Young 1944).

9.4 Conclusions

In the current study the muscle weight was consistently less in the experimental muscle than in the contralateral control, the minimum diameter of the muscle fibres was generally less in the experimental muscle and there was a greater proliferation of connective tissue in the experimental muscles than in the contralateral controls. Each of these characteristic changes was most marked at 50 days after operation and was minimal by 300 days after operation. The reversal of these atrophic effects is indicative of successful reinnervation of the muscle by 300 days after operation.

The muscle fibres recovered from the different atrophic effects at different rates. The level of muscle fibre atrophy, as measured by minimum fibre diameter and the form factor, was minimal at 50 days after operation and very similar by 300 days after operation indicating that the majority of reinnervation had occurred

before the first postoperative observation at 50 days after operation. This is in direct contrast to the recovery of muscle weight of the experimental muscles which had reached only 50 - 60 % of the contralateral control weight by 50 days after injury and repair but 85 - 95% by 300 days after operation. This was true for all experimental groups with the exception of those animals injured by means of a nerve crush. In general, after a nerve crush injury the atrophic effects were less marked. There was a greater and more rapid recovery of contralateral control muscle weight, fewer fibres with pathological features, little or no proliferation of connective tissue and no change in the distribution and relative proportion of each fibre type present. Each of these features was more marked after an injury which involved the transection of the nerve, although the method of repair appeared to be of little consequence. Certain features of denervation, such as decrease in muscle weight, decrease in minimum fibre diameter and the incidence of fibres with pathological features tended to be more marked after repair with a muscle graft, however other features were unaffected by the method of repair. Considering the overall effects of denervation and reinnervation on the target muscles no one method of repair could be said to be consistently better or worse than another. The slow soleus muscle was generally affected to a greater degree than the fast EDL, with a lower level of recovery of muscle weight, a greater level of muscle fibre atrophy, a greater proliferation of connective tissue and a greater change in the relative proportion of each fibre type present.

Thus the effect of the degeneration/regeneration process, on the two target muscles, is defined primarily by the nature of the injury rather than on the method

of repair. After transection of the nerve and repair by any of the three methods used in the current study there was a reversal of most of the atrophic effects and the muscle fibre architecture returned towards that seen in the contralateral control, although the recovery was never complete. The only feature of denervation and reinnervation which failed to be reversed was the change in the distribution and relative proportion of each fibre type present. The change in the relative proportion of each fibre type present would have consequences on the subsequent function of the muscle. The clinical consequences are discussed in chapter 10.

10. General discussion

When a peripheral nerve is injured, to produce either axonotmesis or neurotmesis, the distal portion of the nerve undergoes Wallerian degeneration, as a consequence of being separated from its cell body. The distal segment of the nerve remains excitable for 4 to 7 days after the nerve lesion although there is an immediate loss of motor, sensory and autonomic function, as a consequence of conduction failure across the site of the lesion (Bosch and Mitsumoto 1991). The loss of these functions present as paralysis of the muscles and anaesthesia/analgesia of the area of skin supplied by the transected nerve. Additionally many patients complain of severe pain and of sensations such as tingling, pins and needles or burning (paraesthesia) (Seddon 1975). There is an absence of sweating in the denervated area of skin whereas the area of innervated skin surrounding this tends to experience excessive sweating (Seddon 1975). After the transection of certain nerves there is also an associated change in the temperature, colour and structure of the skin (Seddon 1975).

Many of these uncomfortable symptoms are alleviated once successful reinnervation of the target organs has taken place, although the primary goal of the surgeon is to achieve a degree of useful motor function and protective sensory function. Functional recovery is often incomplete or absent after the repair of transected peripheral nerves (Brushart 1988). Fine coordination is impaired and individual muscles no longer contract independently of one another (Brushart and Mesulam 1980). The restoration of normal sensation after peripheral nerve repair is

unusual (Brushart et al 1981). False localization of sensory stimuli is a common clinical finding after peripheral nerve regeneration and probably reflects an organizational defect in the reinnervation process (Brushart et al 1981) - considered later. There is an obligatory link between normal motor function, its control and its coordination. Any degree of recovered motor power is virtually useless if it cannot be controlled and co-ordinated appropriately. Patients who have undergone peripheral nerve repair complain more often about loss of fine control and coordination and the sensory disturbance than about the loss of power (Myles et al 1992). Reviews of the clinical literature show that only 25% of patients fully recover motor function and only 1 to 3% fully recover normal sensation (Dellon 1981, Mackinnon and Dellon 1988).

The return of useful sensory and motor function depends on the survival of the nerve cell body, the number of fibres reaching peripheral targets, the speed with which new fibres reach the periphery so that degeneration of the end organs does not occur, the maturation of the new fibres to achieve functional competence and the appropriateness of the connections made (Lundborg 1988, Myles and Glasby 1991). Most of these factors cannot be influenced by surgical intervention although the result can be influenced by the timing of the repair, the way the surgeon handles the nerve, the method of repair chosen and the post-operative re-education and rehabilitation (Lundborg 1988).

The timing of the repair has been a source of great debate for a very long time. The current school of thought favours the use of primary nerve repair as this allows the regeneration of axons through open distal basal lamina tubes before

degeneration changes take place to alter the distal internal anatomy (Lesavoy 1991). Although primary nerve repair is the ideal it may not always be possible if there is a significant loss of soft tissue around the injury, if the wound is contaminated, if there is skeletal instability or if the patient is not fit for anaesthesia (Lesavoy 1991). It is important to ensure that the condition of the wound and of the patient is optimal (Lundborg 1988). The way in which the surgeon handles the nerve can have serious consequences on the recovery attained. Careless handling may result in further damage to the nerve which will reduce rather than enhance recovery. It is important to ensure that handling is kept to a minimum, is restricted to the epineurium or perineurium, that the nerve is not put under tension during the repair and that the blood supply is preserved as much as possible.

One of the main aims of all nerve repair procedures is to guide the regenerating axons back to their original end target organs (Harsh 1991), as the regeneration of proximal axons into inappropriate distal pathways is a possible reason for the failure of functional recovery (Brushart 1988). The regeneration of motor axons down Schwann cell tubes that lead to sensory end organs and of sensory axons to motor end plates leads to problems which are two fold. Not only will the axons fail to make functional connections but they may exclude the appropriate axons from the pathways they occupy (Brushart 1988). The degree of mismatch can be influenced by the method of repair implemented - see chapter 1.3. The current study indicates that although the different methods for the surgical repair of peripheral nerves have an effect on the accuracy of reinnervation of the target muscles, there is no corresponding relationship between the method of

repair and the number of associated motoneurones. The number of motoneurones associated with the EDL after the transection of the sciatic nerve was significantly less than normal but there was no significant difference in the number of motoneurones labelled after each of the methods of repair. After injury by means of axonotmesis there was no significant difference in the number of labelled motoneurones compared to normal. Similarly, in a study of the regeneration of sensory neurones, Myles et al (1992) found that the number of labelled cells in the dorsal root ganglia was not significantly different from that seen after other methods of repair. These results suggest that the extent of cell loss is related to the process of nerve section and degeneration rather than to whether successful regeneration takes place. There was no significant difference between the mean minimum cell diameter of labelled motoneurones in the ventral horn of the spinal cord after each of the methods of repair. This was in direct contrast to the results of the mean minimum cell diameter of labelled cells in the dorsal root ganglia, where nerves repaired by grafting were significantly different from those repaired by means of a direct epineurial suture (Myles et al 1992). This result was explained by the fact that atrophy is dependent on whether appropriate peripheral connections are made. Since grafting increases the likelihood of mismatch between axons and appropriate endoneurial tubes, there is likely to be a greater degree of atrophy. If this is indeed the reason behind the difference in mean minimum cell diameter then it suggests that regeneration and reinnervation of motor nerve fibres is more accurate than that of sensory fibres. This finding is in agreement with the work of Brushart (1988) who found a preferential reinnervation of motor branches

by motor axons, regardless of mechanical axon alignement. Although the majority of motor axons reinnervate motor branches this specificity was by no means absolute and many also entered the sensory branches. However, by comparison, an even greater proportion of regenerating sensory axons entered the motor branches, suggesting that sensory axon behaviour is more random. A series of experiments by Brushart indicated that there is an initial random reinnervation of the distal stumps by both sensory and motor axons (Brushart 1991). Specificity is then achieved by the dying off of motor axons which are in sensory pathways while those in the motor pathways are preserved. This pruning is due to a trophic interaction between the motor axons and the motor pathway and/or end organ. There is little or no evidence of topographical or end organ specificity in sensory axon regeneration (Brushart 1991).

There is an alteration in the architecture of the fibres of target muscles after the injury and repair of the sciatic nerve. These changes are more marked after the transection of the nerve as opposed to a less severe crush injury, however there was little or no difference in the architecture between the groups which had been repaired by the different methods. One of the most striking features following the transection and repair of the sciatic nerve was the appearance of groups of fibres of the same type as opposed to the random distribution seen in normal muscle or indeed in muscle which has been reinnervated after a nerve crush injury. The presence of fibre type grouping arises as a consequence of denervated muscle fibres being reinnervated by collateral sprouts from the nerves supplying adjacent muscle fibres. This mechanism acts to ensure that any shortfall in the number of

regenerating nerve fibres is compensated for and does not result in a shortfall in the number of reinnervated muscle fibres. Thus after the transection and repair of a peripheral nerve, the majority of muscle fibres are reinnervated either directly or as a consequence of collateral sprouting. Since the type of muscle fibre is dependent on the nerve which innervates it, reinnervation by collateral sprouting results in the conversion of a great many muscle fibres from one fibre type to another. Indeed, in many cases in the current study, the soleus muscle changed from being composed primarily of type I fibres in normal animals to predominantly type II fibres after the transection and repair of the sciatic nerve. This will have consequences on the recovery of function as explained in chapter 9.2.

Neither the number, size nor distribution of the motoneurones associated with a target muscle or the target muscle fibre architecture differs after the different methods of repairing a peripheral nerve. Despite this there is a difference in the level of nerve function and nerve morphology after the different types of peripheral nerve repair. Myles and Glasby (1991) found that repairing a nerve by the insertion of a graft resulted in poorer recovery of function than after a direct epineurial suture, although there was no difference in the results after the two methods of grafting. Thus the main problems of functional recovery found after peripheral nerve repair such as muscle weakness, uncoordinated movement and the false localization of stimuli is not a consequence of changes in the number of spinal motoneurones, the degree of reinnervation attained or of changes in the muscle fibre architecture. These are not unreasonable assumptions since it was stated by Sharrard (1955) that in cases of poliomyelitis, a considerable proportion of motor

cells may be damaged or destroyed with no signs of clinical paralysis. Similarly the lack of power in a specific muscle can go unnoticed for prolonged periods due to the overlap in function of different muscles and the ability of muscles to compensate for one another to a limited degree - such deceptive muscle action is discussed more fully by Seddon (1975).

It is likely that the limited motor and sensory function recovered after the transection and repair of a peripheral nerve is a result of the inaccuracies which arise in the process of reinnervation. The degree of mismatch between the regenerating proximal stumps and appropriate endoneurial tubes is likely to be one of the most important factors in the recovery of appropriate function. Undirected growth of proximal axon sprouts leads many axons to the wrong muscles so that a pool of motoneurones which previously served one muscle will subsequently control motor units scattered among several (Mark 1969). Hence demands for the contraction of the original muscle will result in the weak contraction of the whole group (Brushart and Mesulam 1980). Similarly the muscle will be reinnervated by the correct sensory axons as well as those formerly serving the skin, leading to the false localization of painful stimuli (Brushart et al 1981).

Great advances have been made in peripheral nerve repair in recent years, however the results in terms of functional recovery are very poor. The results of the current study, considered together with previous literature, clearly show that the main problem in the recovery of controlled and co-ordinated motor function is the lack of accuracy in reinnervation. The method of repair influences this accuracy to a limited degree and trophic interaction plays a part in the natural selection of

specificity. However these mechanisms do not go far enough. The selective reunion of motor and sensory fascicles of a severed mixed nerve may greatly enhance the level of motor function subsequently recovered. Currently the most reliable and simple method for marking motor fascicles is the use of acetylcholinesterase histochemistry however this is such a time consuming process as to make it impractical. Szabolcs et al (1991) reduced the time involved in this process from 28 hours to 4 hours, however 4 hours is still an unacceptable period of time to interrupt a surgical operation. If this process could be performed more rapidly, and personal trials suggest that it could, then it may be a feasible way in which to improve the accuracy of reinnervation and hence lead to better recovery of function. Brushart (1991) suggested the ideal solution to the problems of specificity after nerve repair, which would be to identify and block the stimuli which trigger Wallerian degeneration thus axonal fusion with the preservation of specificity would become a reality returning normal function within weeks of nerve transection. However until such methods become a possibility there seems little expectation of improving upon the current results of peripheral nerve repair.

Appendix 1

Horseradish peroxidase Solutions.

1. Horseradish peroxidase solution for injection.

Horseradish peroxidase crystals (Sigma type vi)	10mg
0.09% NaCl pH 7.4	50µl

2. Perfusion.

a. Vasodilator

Sodium nitrite	1g
Distilled H_2O	100ml

b. Fixative (1.25% glutaraldehyde & 1% paraformaldehyde pH 7.2-7.4)

Paraformaldehyde	25g
Distilled H_2O	250ml
1 N NaOH	dropwise
25% glutaraldehyde	125ml
Distilled H_2O	to make up to 1250ml
0.2 M phosphate buffer	2500ml

Dissolve paraformaldehyde in 250ml dH_2O at 70°C. It is imperative to keep the solution in a fume cupboard whist it is being heated. Add the 1 N NaOH dropwise until the solution clears. Cool the solution to 40°C before adding 125ml 25% glutaraldehyde and making the total volume up to 1250ml with dH_2O. Add 1250ml 0.2 M phosphate buffer to make a final volume of 2500ml. Check pH 7.2-7.4 and store at 4°C. 500ml fixative required per rat.

c. 0.2 M phosphate buffer (pH 7.4)

Sodium dihydrogen orthophospate (NaH_2PO_4 - H_2O)	7.8g
dH_2O	250ml

Dissolve sodium dihydrogen orthophospate in dH_2O.

disodium hydrogen orthophospate (Na_2HPO_4)	32.5g
dH_2O	1000ml

In a separate beaker dissolve disodium hydrogen orthophospate in dH_2O.

Mix the two solutions to obtain 1250ml of 0.2 M phosphate buffer pH 7.4. Store at 4°C.

d. 10% Sucrose buffer

Sucrose	50g
0.2 M phosphate buffer (pH 7.4)	500ml

Dissolve sucrose in phosphate buffer and store at 4°C.

3. Tissue sectioning and storage

The tissue is sectioned and the sections floated out in phosphate buffered saline (PBS).

sodium dihydrogen orthophospate	1.3g
dipotassium hydrogen orthophospate	7.6g
sodium chloride	7.2g
dH_2O	1000ml

Dissolve chemicals in dH_2O, check pH 7.4 and store at 4°C.

4. Enzymatic reaction.

a. Solution A.

Sodium nitroferricyanide	200mg
0.2 M Acetate buffer pH 3.3	10ml
dH_2O	187.5ml

Dissolve sodium nitroferricyanide in dH_2O and acetate buffer.

b. Solution B.

3, 3, 5, 5 tetramethylbenzidine (TMB)	10mg
Absolute alcohol	2.5ml

Heat solution to 37 - 40°C to dissolve TMB in absolute alcohol.

Solutions A and B should be mixed immediately before use to obtain the pre-incubation solution. The solutions should be less than 2 hours old. If artifact is a problem then the amount of sodium nitroferricyanide should be reduced to 100 -160mg.

c. 0.2 M Acetate buffer pH 3.3

Sodium acetate (Mwt 136.1)	2.72g
dH_2O	80ml
Glacial acetic acid	16 - 20ml

Dissolve sodium acetate in dH_2O and add the glacial acetic acid until pH =3.3

d. Peroxide solution

30% H_2O_2	1ml
dH_2O	99ml

Make the solution immediately before to use.

5. Dehydration, clearing and mounting.

a. Gelatin and Chrome alum for coating slides.

Gelatin	10g
dH_2O	500ml
Chrome alum	1g

Heat dH_2O to dissolve the gelatin then add the chrome alum. Cool and filter prior to coating alcohol cleaned slides.

b. Neutral red counter stain.

Acetate buffer

0.1 N Acetic acid	500ml
0.1 N Sodium acetate	750ml

Mix the two solutions together and check pH 4.8

Neutral red stain

Acetate buffer	40ml
Neutral red	10g
dH_2O	1000ml

Mix and filter prior to use.

c. Dehydration and clearing.

All sections should be dehydrated and cleared in a series of graded alcohols and xylene.

70% ethanol	20s
96% ethanol	20s
Absolute ethanol	20s
Absolute ethanol	30s
Xylene	5min
Xylene	5min

Coverslips were applied to slides using DPX mounting medium.

Appendix 2

Correction Factor for split cells - Abercrombie 1946

Fragmented nuclei introduce errors into the assessment of cell numbers since the nuclei may lie partly within two adjacent sections. It is only if the mean dimension in the direction at right angles to the section is minute in relation to the section thickness that this overestimate is negligible (Abercrombie 1946). Abercrombie devised a correction factor to minimize overestimation caused by nuclear fragments.

The formula for the correction factor was

$$P = A \times \frac{M}{L + M}$$

where: P = the true number of cells

A = the number of cells counted

M = section thickness

L = mean cell diameter

Thus the corrected value is a proportion of the crude count taking into account the section thickness and the particle size. If these variables are ignored then large errors are introduced. It is important to realize that the mean nuclear length measured is always going to be less than the true nuclear length since many of the measurements made will be of nuclear fragments. Abercrombie introduced a secondary formula which acts as a correction factor for this. It is based on measurements taken from sections at right angles to those used for the main study.

The secondary formula was

$$L = L - \left(\frac{0.21 \times T}{T + N} \times L \right)$$

where: L = true mean cell diameter

T = measured mean cell diameter

N = section thickness

Hence it is important to calculate the true value of L from sections taken at right angles to those used in the main study and using the corrected value of L in the first formula.

APPENDIX 3

(1) Toluidine Blue

Method

1. Stain unfixed sections for approximately 1 minute.
2. Wash briefly in tap water then differentiate in 50% alcohol until excess die is removed.
3. View specimen.
4. If required dehydrate, clear and mount in D.P.X.

Solutions

1% Toluidine blue in 1% borax (disodium tetraborate) in water. Filter before use. Keeps well.

Results

Stains tissue components blue and is used to check orientation and preservation of during sectioning.

(2) Haematoxylin and Eosin

Method

1. Stain unfixed sections in Harris's Haematoxylin for 1-3 minutes depending on the freshness of the stain and the tissue. Normally approximately 1 minute but judge by eye as sections should be very pale lilac.
2. Blue by washing in warm tap water for 5 minutes.
3. Counter stain in Eosin for 1 minute.
4. Wash in cold tap water.
5. Dehydrate, clear and mount in D.P.X.

Solutions

(a) Harris's Haematoxylin

Harris's alum Haematoxylin	5g
Absolute Ethanol	50ml
Ammonium or Potassium Alum	100g
Sodium Iodate	1g
Glacial Acetic Acid	40ml
Distilled water	- make up to 1.25 litres

Dissolve Haematoxylin in alcohol and add to alum solution. Bring to the boil, allow to cool and then add glacial acetic acid. Boil for 2 minutes and add sodium iodate. Allow to cool and filter. Keeps well but filter before use.

(b) Eosin

Eosin (Alcohol soluble)	5g
Absolute Alcohol	350ml
Distilled water	150ml

Results

Stains nuclei and basophilic material dark blue and other cytoplasm pink. It is used to assess muscle fibre architecture, fibre splitting, position of nuclei, presence of vacuoles, signs of fibre regeneration etc. Hyaline fibres are conspicuous, staining dark pink.

(3) Modified Gomori's Trichrome

Method

1. Stain unfixed sections in Harris's Haematoxylin for 1minute.

2. Rinse in distilled water.

3. Stain in Modified Gomori's Trichrome for 2 hours at room temperature.

4. Differentiate in 2 very quick changes of 0.2% Acetic Acid.

5. Dehydrate, clear and mount in D.P.X.

Solutions

Gomori's Trichrome	
Chromotrope 2R	3g
Fast Green FCF	1.5g
Phosphotungstic acid	3g
Distilled water	500ml

Results

Ragged red fibres stained bright red.

(4) Masson's Trichrome

Method

1. Stain unfixed sections in Harris's Haematoxylin for 30 seconds.

2. Blue by rinsing in running tap water for a few minutes.

3. Differentiate in Picric Acid for 15 seconds.

4. Wash in running tap water.

5. Stain cytoplasm red in Ponceau 2Rfor 2.5 minutes.

6. Wash in running tap water.

7. Differentiate and mordant by immersion in PMA for 5 minutes.

8. Wash in running tap water.

9. Counter stain connective tissue fibres in Fast green (FCF) for 15 seconds.

10. Wash in running tap water.

11. Dehydrate, clear and mount in D.P.X.

Solutions

(a) Harris's Haematoxylin - as for Haematoxylin and Eosin.

(b) Picric Acid - 1% Picric Acid in 70% alcohol. (CAUTION!)

(c) Ponceau - 1% Ponceau de Xylidine (Ponceau 2R) in 0.5% Acetic Acid.

(d) PMA - 2% Phosphomolybdic Acid in distilled water.

(e) Fast green - 0.5% Fast green in distilled water.

Results

Muscle fibres stain red/brown, nuclei stain blue/black, connective fibres stain green. Shows muscle fibre architecture and connective tissue proliferation.

(5) NADH-TR (Nicotinamide Adenine Dinucleotide Tetrazolium Reductase)

Method

1. Incubate unfixed sections for 30 minutes at 37°c - sections go black.

2. Rinse gently in distilled water (can store in dH_20 until ready to mount).

3. Mount in Uvinert.

Solutions

(a) Tris Buffer (0.2M pH 7.4)

Tris (hydroxymethyl methylamine)	12..1g
HCl (normal)	82.8ml
Distilled water	make up to 2 litres

Add HCl gradually until appropriate pH is reached - usually approximately 67ml

(b) Incubation Medium

Tris Buffer 0.2M pH7.4	60ml
NADH	16mg
Nitroblue Tetrazolium	20mg

Mix and filter immediately before use. The solution should have a black tinge prior to filtration.

Results

Intermyofbrillar material (mitochondria, sacroplasmic reticulum, t-tubules) stains dark blue. Can be used for fibre typing - type I dark, type II intermediate, type IIb pale.

Shows proliferation of mitochondria, cores etc.

(6) SDH (Succinate Dehydrogenase)

Method

1. Incubate unfixed sections for 30 minutes at 37^{o}c or overnight at room temperature.

2. Rinse in distilled water..

3. Mount in Uvinert.

Solutions

(a) Incubation Medium

Sodium Succinate 0.1M (0.27gper10ml)	100ml
Formdimethylamide	100ml
Phosphate buffer 0.1M pH 7.6	100ml
Nitroblue Tetrazolium	200mg

(b) Phosphate Buffer 0..1M pH 7.6

Sodium dihydrogen phosphate 0.2M	3.25ml
diSodium hydrogen phosphate 0.2M	21.7ml
Distilled water	25ml

(c) Sodium dihydrogen phosphate 31.20g

Distilled water	1 litre

(d) diSodium hydrogen phosphate 28.39g

Distilled water	1 litre

Results

SDH stains the intermyofibrillar and subsarcolemmal mitochondria blue.

Can be used for fibre typing and to show excess of mitochondria.

(7) Myosin Adenosine Triphosphatase (ATPase)

Method

1. Air dry unfixed sections for 2 hours minimum.
2. Pre-incubate at room temperature - 15 minutes for acids (pH 4.35 and 4.6) and 20 minutes for alkali (pH10.2).
3. Wash 3 times in tap water and 3 times in distilled water.
4. Incubate at 37°c. Acids - place incubation medium in coplin jar and place in incubator for 45 minutes. Alkali - place incubation medium in coplin jar and microwave on low power for approximately 45 seconds or until reaches 37°c, add slides and incubate at 37°c for 30minutes.
5. Wash in 2 changes of 1% calcium chloride.
6. Place in 2% cobalt nitrate for 3 minutes.
7. Quickly wash in 3 changes of tap water and 3 changes of distilled water.
8. Rapidly transfer to developer - 1% ammonium polysulphide - and leave for 5 minutes. Sections should immediately go black. Speed is essential at this stage, if too slow get poor results. This stage must be done in a fume cupboard!!
9. Wash well in tap water and then distilled water.
10. Mount in Uvinert.

Solutions

(a) Pre-incubation solutions

pH 4.6

Sodium Acetate 0.2M	30ml
Acetic acid 0.2M	30ml

Adjust to pH 4..6 with 1M Sodium Acetate.

pH 4.35

Sodium Acetate 0.2M	18ml
Acetic acid 0.2M	42ml

Adjust to pH 4..6 with 1M Sodium Acetate.

pH 10.2

Tris Calcium Buffer	60ml

pH should be 10.2.

(b) Incubation solution

ATP (diSodium salt)	300mg
Tris Calcium Buffer	200ml

Adjust to pH 9.5 with N/10 KOH or HCl

(c) Tris Calcium Buffer

Tris (hydroxymethyl) methylamine	6.05g
Calcium Chloride (MW 147)	1.32g
Distilled water	make up to 500ml

Make up fresh before use.

(d) 1% Ammonium Polysulphide

Make up immediately before use. It is very pungent so must always be used in fume cupboard. Solution should be very pale yellow - if it is bright yellow then it has gone off.

(e) 0.2M Acetic Acid

Acetic Acid	11.6ml
Distilled water	1litre

(f) 0.2 M Sodium Acetate

Sodium Acetate	27.21g
Distilled water	1litre

(g) 0.18M Calcium Chloride

Calcium Chloride	16.46g
Distilled water	1 litre

(h) 1% Calcium Chloride

(i) 2% Cobalt Chloride

Solutions (e) - (i) made up in stock solutions and stored at 4^{o}c for up to one month.

Results

It is used to differentiate muscle fibre types. Serial sections stain differently according to pH of pre incubation solution.

Fibre Types	I	IIA	IIB	IIC
pH 4.35	*dark*	*pale*	*pale*	*dark*
pH 4.6	*dark*	*pale*	*intermed*	*dark*
pH 10.2	*pale*	*intermed*	*dark*	*dark*

Further details of all histological and histochemical staining techniques can be found in Cumming et al (1994).

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